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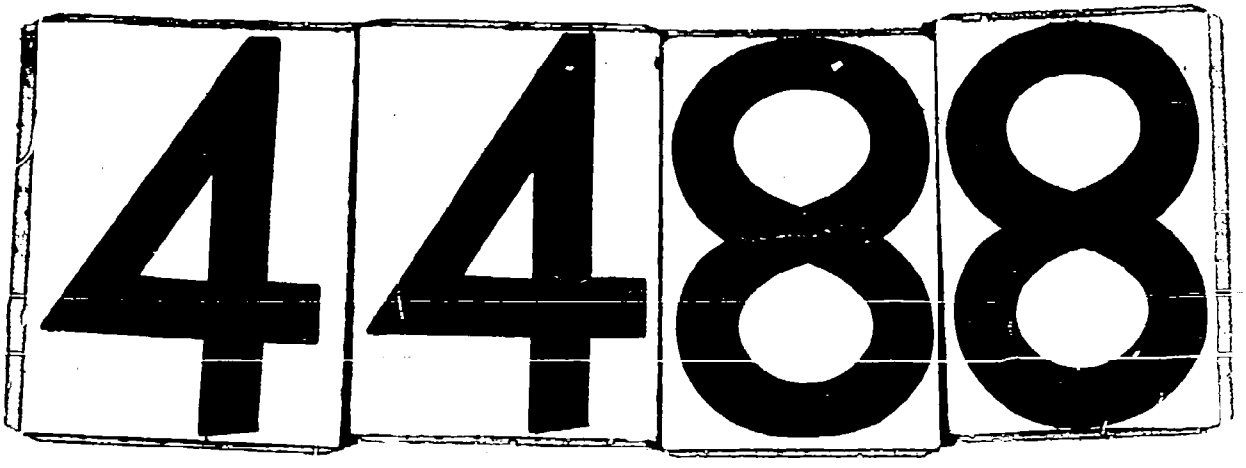

UNCLASSIFIED

LOBUND - ONR
Semi-Annual Progress Report
Contract N6-ori-33, Task Order III

1 July 1952 - 31 December 1952

TO: Chief of Naval Research
Office of Naval Research
Department of the Navy
Washington 25, D. C.

ATTN: Microbiology Branch, Code 443

FROM: J. A. Reyniers, Principal Investigator
N6-ori-83, Task Order III

SUBJECT: Enclosed Semi-Annual Progress Report (1 July 1952 to 31 December 1952). Contract N6-ori-83, Task Order III NR:131-067

DATE: 13 February 1953

Sir:

I am herewith submitting the LOBUND-ONR Semi-Annual Progress Report for the period 1 July 1952 to 31 December 1952.

As a result of conclusions reached at a conference on germ-free life held under the auspices of the NRC at which ONR was represented by Capt. C. W. Shilling, Deputy for Bio Sciences and Dr. Roger Reid, Head of the Microbiology Branch, it is now necessary to set into operation plans for the expansion of LOBUND Institute. Consequently a reorganization of staff and objectives will take place in the next period. The reorganization is primarily aimed at firming up the base for germ-free life production. During this period and as rapidly as possible additional equipment will be planned, constructed and put into operation to the limits of present housing facilities. This will permit among many things training of additional personnel and expansion of collaborative activities.

As presently planned the basic LOBUND Institute program on germ-free life will continue as a group effort. This program has been discussed in earlier reports but those aspects dealing with rearing of new forms, description of germ-free forms now available in numbers, and development and testing of germ-free systems will be emphasized. Especially it may be desirable to extend the descriptive survey by setting into operation a program in physiology and pharmacology. Specifically it is planned to throw into this effort the facilities of what has been our Biological Engineering program into order to provide greater facilities for the testing programs. It is hoped that the exploratory programs will be continued but it is evident that they may decrease in the interim between this report and expansion which must take place.

The report submitted points out that we are now past the 6th generation of germ-free rats thus settling once and for all the old problem of the necessity of microbes to life as represented by the white rat. Moreover, this also establishes a landmark in that there can no longer be any doubt relative to our ability to maintain a colony of these animals. The only problem is the expansion of facilities to make more of these animals available for experiment. With such an expansion the cost of germ-free rats is reduced to a reasonable figure making this valuable tool available for research. This is in itself complete vindication of our original position as set out in 1928.

It is very essential that the basic survey be continued in biochemistry if the germ-free animal is to be used successfully. While this survey of nutritional needs may seem plodding to those who champ at the bit in their desire to use the animals experimentally, long experience has convinced me of the absolute necessity of this approach. The systematic approach which characterized the work in LOBUND Institute will continue to be emphasized.

Sincerely yours,

James A. Reyniers

James A. Reyniers
Principal Investigator of
Contract N8-ori-83, Task Order III

University of Notre Dame

LOBUND Institute-ONR Semi-Annual Report

13 Feb. 1953

For Period 1 July 1952 to 31 December 1952

CONTRACT: N8-ori-85, T. O. III NR:131-067

Prof. James A. Reyniers, Principal Investigator

Director, LOBUND Institute

Sections Reported

I. ADMINISTRATIVE

II. GERM-FREE LIFE PRODUCTION

III. BIOCHEMISTRY AND NUTRITION

IV. BACTERIOLOGY AND SEROLOGY

V. PHYSIOLOGY AND PATHOLOGY

VI. VIROLOGY

VII. COLLABORATIVE PROGRAMS

VIII. SUMMARY

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I. ADMINISTRATIVE

FROM: R. F. Ervin, Assistant Director for Administration
(With the assistance of B. Eilerman and J. Mahon)

TO: J. A. Reyniers, Director

SUBJECT: LOBUND-ONR Report, 1 July 1952 - 31 December 1952

DATE: 31 January 1953

A. Task Personnel:

As of 1 July 1952, 48 persons were employed on this Task Order. The salaries of 39 were paid from contract appropriations and 9 were paid by the University of Notre Dame. On 31 December 1952 50 were employed. In this six month period a total of 12 people were hired on the Task. Therefore, in order to increase total employment by 2, 12 persons were hired. This indicates continued job turnover is high. However, it is limited to a very small percentage of the total staff positions. While we have attempted in several ways to reduce this turnover, it becomes more and more apparent that a major re-evaluation of salary scales on certain jobs must be made. For example, the diving job in connection with the GF colony tank operation is critical and cannot afford frequent turnover.

B. Physical Plant:

Major revisions are being made to our GF air supply systems. New blowers are being installed and emergency power sources are being investigated. This is in keeping with our efforts to make the germ-free system foolproof and immune to emergency breakdowns if possible. The University has spent considerable money on the air conditioning systems to make their operation efficient and continuous.

Because of expansion of control animal work, it has been necessary to convert the Quonset animal house from a "disease-free" colony operation to a standard experimental animal building. This was done reluctantly and we hope for a limited time only. Steps are being taken now to acquire from the University a portable building for our control chick experiments.

The new histology section has been completed and is now functioning in about 1/3 of the X-ray room. This has given better facilities for histology and at the same time permitted more space for the expanding physiological work in Dr. Gordon's laboratory.

Our media production and glassware cleanup room has been rearranged in an attempt to keep up with increased demand. An automatic glassware washer is being purchased to help speed up this operation.

Desk space for Drs. Martin Forbes and Edgar Hawk has been provided by crowding and rearranging the egg incubation room. Forbes is on the Army necrosis problem and Hawk is on the NIH vitamin problem (see Collaborative Programs below and in Biochemistry Section III).

C. Publications:

Our work on the collaborative dental caries paper with Zoller Clinic is now completed. Discussions are to be held soon between the Koller group and the editor of the J. of Dental Research.

Considerable data found at LOBUND Institute during the course of the survey project on GF life have been given to Dr. Norbert H. Fell for inclusion in the Handbook of Biological Data being published under the auspices of the NRC.

Fair progress is being made on LOBUND Reports No. 3. It is hoped this will issue within the next six months.

D. Major Collaborative Programs:

These are discussed in detail in the various research and collaborative sections of this report but are listed here as follow:

AEC (Advisory Committee) - Radiation injury
Army Med. Corps (Paul Gyorgy) - Liver necrosis
NIH (Floyd Daft) - Vitamin C
NIH (W. H. Wright) - Amebiasis
Zoller (J. Roy Blayney) - Dental caries

E. Proposals and Contract Status:

A proposal was submitted to ONR Washington on 19 November 1952 for extension of Phase III from 1 January 1953 to 31 December 1953. The amendment authorizing this has not been received to date (30 January 1953).

Amendments extending Phases I and II to 30 June 1953 are now in force. Proposals for extensions of these phases will be submitted as quickly as the problem of accumulated unobligated funds is solved by extension amendments. An early meeting is planned at LOBUND Institute between an ONR Washington contract negotiator, a representative from contracts administration, ONR Chicago, and LOBUND Institute administrators.

F. Government Furnished Equipment and Material:

A list of needed equipment and material has been forwarded to ONR Chicago with the request that a search be made to determine their availability as GFE. This list is included herewith.

Apparatus

1 International clinical table model centrifuge 115 V. A.C. with two interchangeable heads (4 place for 15 ml tubes and 4 place for 50 ml tubes). Similar to Sargent #16225.

1 International refrigerated centrifuge Model PR-1 like Sargent #S-16696 or the same type without refrigeration (Sargent 16600)

1 Analytical balance with chainomatic device. Either Ainsworth or Christian-Becker. 200 grams capacity, 1/20 mg at full load sensitivity. Like Sargent #S-2588 or S-2615.

1 Constant temperature water bath with inside dimensions approximately 18 x 12 x 6 inches. Like Sargent #S-84745.

1 Warburg respiration apparatus complete with glassware. Like Sargent #S-7480 or S-7486.

1 Portable Potentiometer like Minneapolis-Honeywell Series 126W3V. Calibrated 0-71 millivolts.

Pittsburgh Electrodryer apparatus for dehumidification of air (such as was used on naval vessels when they were prepared for the "mothball" fleet). It is not possible for us to give exact models, etc., but would appreciate any information on availability from Navy stores.

1 Bausch & Lomb Model L photomicrographic apparatus complete with ribbon filament illuminator. Like Sargent #S-68090.

Material

Leaded Brass

2 lengths 3/16" round
" 1/4" "
" 5/16" "
" 3/8" "

Aluminum or Dural

2 lengths each
Rounds - 1/4, 1/2, 5/8, 3/4, 7/8, and 1"
Flats - 1/8 x 1, 1½, 2, 2½, and 3"
5/16 x 1, 1½, 2, 2½, and 3"
1/4 x 1, 1½, 2, 2½, and 3"
3/8 x 1, 1½, 2, 2½, and 3"
1/2 x 1, 1½, 2, 2½, and 3"

Stainless Steel

2 lengths each
Flats - 1/8 x 1, 1½, 2, 2½ and 3"
3/16 x 1, 1½, 2, 2½ and 3"
1/4 x 1, 1½, 2, 2½ and 3"
Hex - 1/2 and 3/4"

II. GERM-FREE LIFE PRODUCTION

FROM: B. A. Teah, Chief of Germ-Free Life Production (With the assistance of E. Zelmer, H. Thompson, B. Werner, J. Uselding, J. Timmons, L. Terry, W. MacAllister, and M. Ziemba).

TO: J. A. Reyniers, Director

SUBJECT: LOBUND-ONR Report, 1 July 1952 - 31 December 1952

DATE: 31 January 1953

A. Apparatus and Techniques:

The changes in the germ-free equipment and techniques to facilitate the handling and production of germ-free animals are listed briefly as follows:

1. Previously reported (ONR Report, January - June, 1952) as being tried and thought to be satisfactory were the all-neoprene gloves formulated and made by the Dewey and Almy Co. After more usage and new shipments, these gloves have not been satisfactory. They were found to tear very easily during sterilization and to possess many small weak spots which decreased their safe use in normal operations.

2. Use of torque wrenches for all sterile-lock tightening is now a standard procedure.

3. A new watering device for keeping cages cool during sterilization of claves designed, and put into use.

4. All diets now prepared for use in GF cages are in weighed amounts so that each sterilization will be similar and the amount of diet used may be more accurately recorded.

5. Wire false bottoms are being introduced into all cages, i.e., these replace sawdust. It is felt that this considerably improves the activity and appearance of the germ-free animals, and eliminates the dampness usually encountered when animals are bedded directly on sawdust.

6. Several types of diet containers were tried in an effort to eliminate wastage by spilling, etc. The two most efficient up to now are: (a) A piece of stainless steel shaped as a quarter circle with upper edge having a 1/4" lip. This is permanently attached to side of the cage. (b) An ordinary ice cube tray, 8" x 3½" x 2" with the movable dividers left in place.

7. An eight compartment metabolism cage, with facilities for collection of urine and feces on filter paper, was designed and is now being tested.

8. Three additional quick-opening traps and one proportioning device (as originally reported in ONR Report, July - December, 1950) were received and placed into operation.

9. All cages are being equipped with individual clave door holders when such are not in use.

10. New type instrument holders were designed and placed in use in our operating cage.

B. Animal Production:

In this reported period the germ-free work was handled with the following equipment: 10 series 200 cages, 10 series 100 cages, 5 series 50 cages, 4 series 20 cages, 1 x-ray cage, 2 examining ceges and 1 operating cage - a total of 33 cages.

The stock colony of rats, a resume of which is given below, has gone into the seventh generation. Reproduction was satisfactory but the percentage of rats weaned was low. It is felt that with the present change of diets from L-128 to L-356, the number of weaned rats should and will show a marked increase.

The chicken work, a resume of which is given below, has been very troublesome and there has been an unusually high number of contaminations. After investigation, it is felt that one cause of these contaminations has been the improper handling of eggs at the source, i.e., the supplier was sanding the eggs which in turn forced the bacteria deep into the pores of the shell.

The guinea pig work for NIH to which two cages were allocated is fully described in the pathology section (Section V). Twenty two Caesarian operations for ten experiments were carried out.

Germ-Free Rat Production
(1 January 1952 - 31 December 1953)

Animals in colony 1 January 1952 - Adult - 8 ♀, 11♂
Preweaned - 18

No. born - 720
Ave. No./litter - 6.4
No. weaned - 258
% weaned - 35.8%
% weaned in control colony* - 24.6%
No. litters born - 112**
No. litters weaned - 42
% weaned/weaned litter - 85%
% weaned/weaned litter - in control colony* - 60%

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*LOBUND Control Animal colony
** Includes 7 litters (49 pups) born from dams on an experimental necrosis diet. Excluding these, the % weaned is 38.5%.

No. weaned animals lost from spontaneous death - 35
No. weaned animals lost from contamination - 37
No. weaned given to experiments - 103
No. weaned in colony 31 December 1952 - 120

Breakdown of experiments receiving weaned rats:

Colony tank apparatus	- 40
X-ray	- 32
Dental Caries	- 19
Necrosis	- 8
Biochemistry	- 4
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Rat Contaminations, Stock and Experimental

A. No. of Cages Contaminated	5
1. Contaminations from Manipulations	3
No. of transfers	78
No. of sterile claves	1200
No. of cage sterilizations	100
Total manipulations	1378
2. Contaminations due to glove break	2

Germ-Free Chick Production
(1 January 1952 - 31 December 1952)

No. eggs set	2971
No. hatch controls	1096
No. hatched in R.G.F. units	395
No. hatched in G.F.	298
No. chickens contaminated from hatch	97
No. exps. contaminated from hatch	11
No. chickens contaminated by manipulations	66
No. exps. contaminated by manipulations	5
No. exps. contaminated by glove breaks	2
No. exps. germ-free	26

Chicken Contaminations

A. No. of Cages Contaminated	18
1. Contaminations from manipulations	5
No. of transfers	20
No. of sterile claves	450
No. of cage sterilizations	70
No. of trap passages	50
Total Manipulations	595
2. Contaminations from Glove Break	2
3. Contaminations from Egg Passages	11

III. BIOCHEMISTRY AND NUTRITION

FROM: T. D. Luckey, Chief Biochemist
(With the assistance of M. Beaver, L. MacAllister, T. Wende, A. Pappas, J. Pleasants and L. Takacs. In collaboration with M. Forbes (Univ. of Penna.) and E. Hawk (NIH) who are in residence at LOBUND Institute)

TO: J. A. Reyniers, Director

SUBJECT: LOBUND-ONR Report, 1 July 1952 - 31 December 1952

DATE: 31 January 1953

A. Biochemistry:

1. Survey of the Germ-Free Chick:

The comparison of the germ-free chick with conventional biochemical chicks is illustrated by the data taken from 4-5 week old White Leghorn chicks as presented in Table I. It is noted that there are no great differences between the two (with the exception of riboflavin in the bile and possibly niacin). The threefold differences found in liver vitamin B_{12}, cecal contents niacin, and biotin in the bile may be of some biological importance. The high amount of riboflavin in the bile very possibly represents a qualitative difference in some liver function.

The values given show statistical difference ($p = 0.05$) for lung ash and fat; brain ash and pantothenate; niacin in the cecal contents; liver ash, fat, pantothenate; folic acid and vitamin B_{12}; and bile riboflavin and biotin.

2. Survey of the Germ-Free Rat:

Since material is still being collected for this project, it is premature to discuss any details. The general pattern appears to be similar to that of the chicks: there are no great differences. In short the germ-free animals are biochemically healthy.

3. Analysis of Rat Milk:

The work with the analysis of rat milk has shown the following mineral composition on a wet weight basis:

Element	%
NA	0.136, 0151
K	0.146, 0.111
Ca	0.251, 0.289, 0.490
P	0.184, 0.227

These values agree fairly well with previous data.

TABLE I

BIOCHEMICAL SURVEY OF FRESH TISSUES IN WHITE LEGHORN CHICKS

Tissue	Gp*	Ave No.	Fat %	Total N_2%	Flavin γ/gm	Niacin γ/gm	Calpan γ/gm	Biotin γ/gm	Folacin γ/gm	Vit. B_{12} mγ/gm	Ash %dry
Blood	A	3	-	-	0.0	2.6	0.9	0.05	0.72	2.86	-
	B	4	-	-	0.0	0.0	1.2	0.06	0.72	4.21	-
P**					1.0	.08	.38	.15	.97	.31	
Lungs	A	4	4.8	2.7	-	-	-	-	-	-	7.5
	B	4	3.4	2.5	-	-	-	-	-	-	6.3
P			.02	.08							.01
Brain	A	3	5.5	-	2.3	25.4	55.3	0.88	0.53	15.1	8.4
	B	4	6.8	-	2.1	21.8	64.8	1.11	0.55	16.3	7.1
P			.27		.70	.19	.01	.16	.80	.74	.03
Bone	A	3	-	-	14.2	32.0	5.9	1.7	6.0	6.0	-
	B	3	-	-	12.8	20.5	5.2	0.9	1.8	3.4	-
P					.80	.52	.58	.23	.63	.61	
Muscle	A	3	2.4	-	8.1	33.5	17.5	3.0	0.8	15.1	5.2
	B	4	2.3	-	10.9	46.5	14.3	2.8	1.1	15.6	4.4
P			.27		.59	.89	.86	.69	.33	.83	.26
Cecal	A	9	-	-	10.9	12.1	5.5	0.2	2.3	14.5	20.3
Contents	B	10	-	-	7.3	37.2	7.7	0.5	1.8	18.4	24.3
P					.09	.01	.68	.66	.26	-	.06
Liver	A	10	4.0	3.1	21.6	10.9	66.4	5.1	4.4	323	5.8
	B	13	7.5	3.5	23.3	10.8	48.0	4.3	3.3	171	4.3
P			.01		.50	.94	.01	.22	.02	.01	.02
Bile	A	3	-	0.72	84.2	7.6	3.1	0.24	0.36	6.0	11.6
	B	3	-	0.70	12.7	8.8	2.3	0.07	0.40	3.9	12.5
P				.93	.01	.85	.27	.01	.94	-	.41

* Group A is germ-free chicks, Group B is conventional chicks. All chicks were 4-5 weeks old.

** Statistical difference indicated by less than 0.05.

Efforts are directed toward obtaining a suitable method for the determination of individual fatty acids in rat milk. The next step in this work will be the quantitative examination of the amino acids of rat milk.

B. Nutrition:

1. Reproduction on Steam Sterilized Diets:

The present germ-free albino rat colony, and a parallel conventional* colony were maintained from 1950 to late 1952 almost exclusively on a semi-synthetic type diet, L-128, containing all known essential nutrients in quantities sufficient to be adequate after autoclaving. It also contained liver and yeast as potential sources of unknown factors. While permitting reproduction through successive generations (now up to the 6th generation) this diet and/or other factors operating in the germ-free environment, resulted in sub-optimal performance, especially in lactation. Fifty-five GF ♀ bore 885 young (16.1 per ♀) but reared only 311 (5.7 per ♀). Those reared were too small to wean at 21 days and were weaned at 26 days, when weighing about 27 gm on the average. The following Table II gives comparative data obtained with diets L-128, for L-109 (another semi-synthetic diet), and for L-189.

TABLE II

Diet	State of Animal	% Weaned	Est. Wt. at 21 Days	Post-weaning Growth gm/day to 70 days old		% ♀♂ bearing at least one litter.
				♂	♀	
128	GF	38	20	5.0	3.6	91
128	Conv.	32	33	5.8	3.4	-
109	GF	0	-	3.5	2.2	33
109	Conv.	72	51	5.1	3.2	100
189	GF	-		4.1	3.0	
189	GF then conv.	100	45			100
189	Conv.	92	35	4.3	2.9	83
Rockland**	Conv.	80	40	3.6	2.6	91
Special Rockland***	Conv.	75	21			100

Since natural type diets might improve performance by providing unknown factors or known factors in better proportion, a long series of experiments were set up to find the best natural type stock diet. Pilot experiments showed that commercial natural-type diets could not be used as such. Autoclaving reduced their growth-promoting power by a third, cut the % weaned by a fourth, and reduced the weaning wt. by over 40%. Two approaches were then followed:

* Contaminated with the usual animal colony contaminants.

** This diet was not sterilized; but is given as an example of performance on a commercial diet.

*** This diet was made by the Arcady Milling Co. and was patterned after Diet L-275 (see Table III).

A natural-type diet was made up at LOBUND with the usual stock diet proportions. This suffered the above-mentioned reduction in efficiency on autoclaving.

A series of experiments showed that the efficiency could not be restored by adding more B-vitamins but was completely restored by raising the proportion of casein. This casein fortified diet became L-189 which was fed through 6 conventional generations, with the results shown in Table II and has been tried several times in GF. Of two GF ♀ which had a chance to breed, neither bred up to 106 days of age while GF, but both conceived within 4 days of being brought out to the conventionally contaminated colony. They reared healthy litters (see Table II).

Various supplements: casein, corn or soya oil, and Torula yeast, were added to a commercial stock ration in various proportions. The diet was fed nonclaved. No one combination gave optimum performance in all functions, but one combination gave better results than the others in birthweight, weaning weight and neonatal survival.

This combination became a pattern for several diets fed to GF rats in late 1952, i.e., L-330, L-349 (L-330 with cellophane). These contained 78% ground Rockland Rat Checkers, 10% crude casein, 7% Torula yeast, 5% soya oil, plus concentrated water and fat-soluble vitamins.

While these experiments were going on, a variety of changes were made in the texture and degree of refinement of the ingredients in the old GF stock diet, L-128, to see if reproductive efficiency and the frequency of twisted ceca would be effected.

Complete results of these GF experiments are not yet available.

TABLE III
Diet Composition (%)

Ingredient	Diet L-189	L-275
Whole wheat flour	50	-
Buttermilk powder	13.5	-
Yeast, dried Brewer's (Torula)	9	7
Alfalfa leaf meal	4.5	-
Meal scrap powder	4.5	-
Salts L-II	2.7	-
Liver powder 1-20	2	-
Oil, corn (soya)	2	5
Ladek-3 (A & D only)	1	1
B-mix 30	2	-
Casein, lebco (crude)	10	10
Rockland Rat diet	-	78

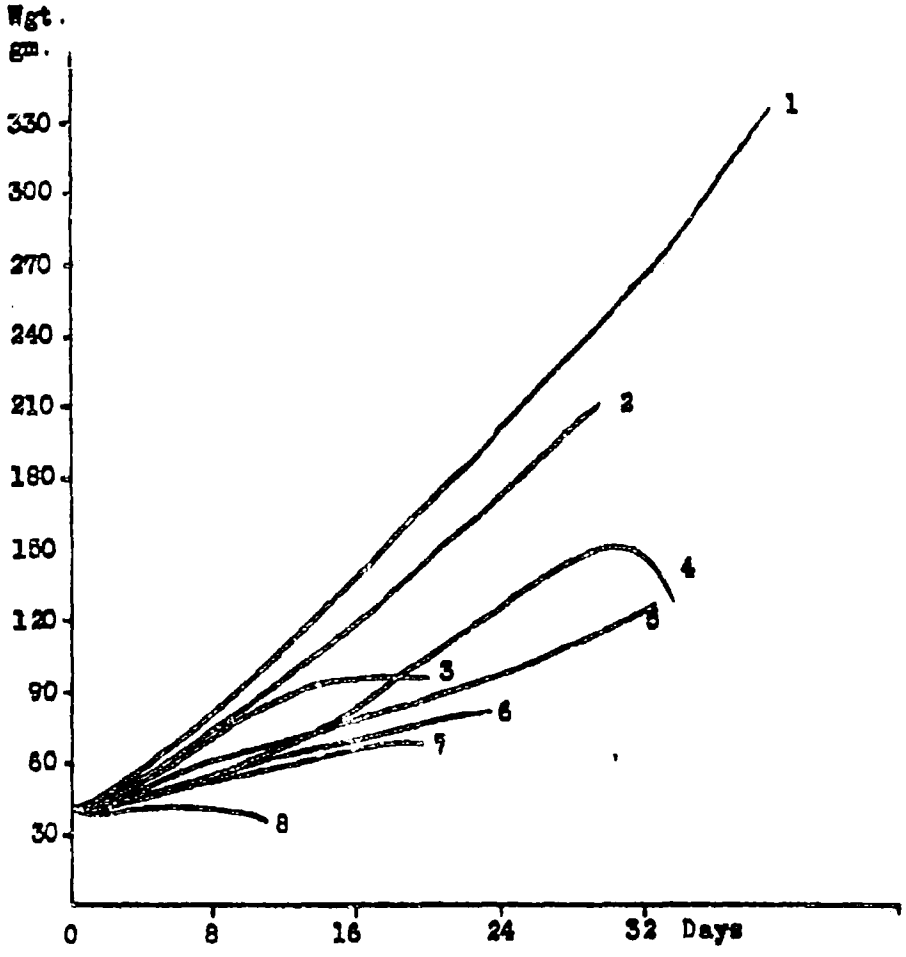
(parenthesis give type material used in Diet L-275)

2. Chick Nutrition Survey:

The growth results from an examination of the vitamin requirement of the germ-free chick are summarized in Figure 1.

Figure 1

Growth of Germ-Free White Leghorn Chicks
Fed Vitamin Deficient Diets



1. Control
2. Vitamin A low
3. Folic Acid low
4. Biotin low
5. Vitamin D low
6. Niacin low
7. Riboflavin low
8. Thiamin low

It is quite evident that germ-free chicks require the same vitamins, qualitatively, as the literature and our work indicates, are needed by the conventional chick. Other data indicates the germ-free chick requires vitamins D, E and K. We are presently working with pantothenic acid and hope to do some work in the future with less well defined vitamins such as vitamin B_{12}, p-amino-benzoic acid, lipoic acid, and possibly vitamin C. The next major study on this problem is an examination of the quantitative requirement of one or more of the B-vitamins.

3. Antibiotics Fed to Germ-Free Chicks.

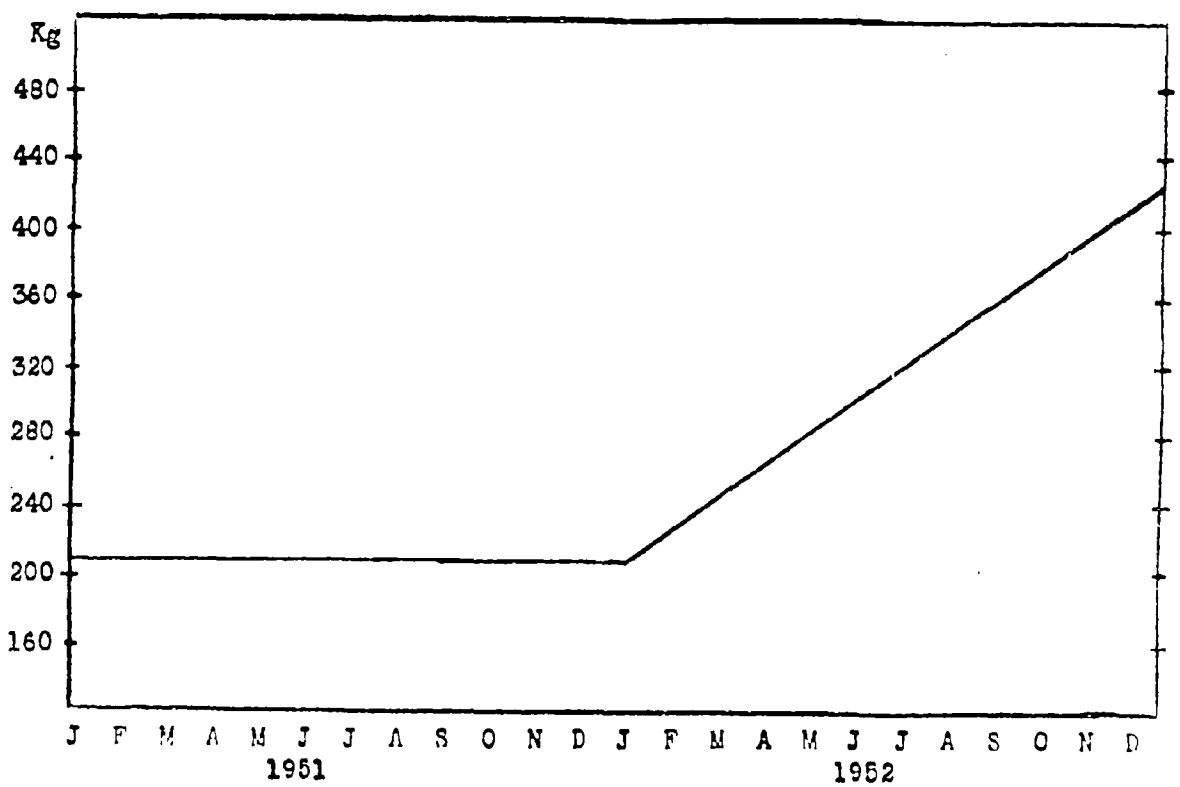
This problem was summarized in some detail six months ago (also a special report was submitted in June 1952). Since this time we have turned to a study of feeding smaller quantities of antibiotics to germ-free chicks and are injecting antibiotics into germ-free turkeys. The work has gone rather slow due to low numbers of chicks hatched and some contamination (see production report).

4. Diet Laboratory

The work of the diet laboratory for the past year is summarized in Figure II. The addition of a second mixing bowl has helped the busy diet girls materially. As shown in the figure, the quantity of diet made each month during 1952 was considerably greater than during 1951. If this increase continues, there will be a great need for more room for this operation.

Figure 2

Amount of Solid Diet Made Each Month ('51-'52)



C. Collaborative Problems:

1. The Etiology of Dental Caries (with Dr. Blayney and Dr. Orland of the Zoller Dental Clinic of Chicago University).

The first phase of this work is completed and the work has been written up for publication. This written material will be submitted as a special report in the near future. The conclusions of the paper are, as previous reports in this section have indicated, that germ-free rats do not develop carious lesions when fed a diet shown to be highly cariogenic in conventional rats.

The second phase is reported in the Bacteriology Section IV of this Progress Report.

2. Etiology of Hemorrhagic Liver Necrosis (with Dr. Gyorgy and Dr. Forbes of the University of Pennsylvania Medical School).

See progress report prepared by Dr. Forbes in Section VII.

3. The Role of Ascorbic Acid (or Antibiotics) Replacing the Rat's Requirement for Pantothenic acid, in collaboration with Dr. Daft and Dr. Hawk of the Institute for Arthritic and Metabolic Diseases at NIH.

Preliminary work is being done to try to set up a critical experiment for the use of germ-free rats.

It seems premature to do more than mention that this work is underway. A progress report from Dr. Hawk who is in residence at LOBUND will be submitted in July.

4. The Presence of Ergothionine in Germ-Free Chick Tissues, by Dr. D. Melville, Cornell University Medical School.

Dr. Melville received samples of germ-free chick red blood cells and has found ergothionine to be present. He was written a paper to report this separately. When reprints or references are obtained, CNR will be notified.

5. The Presence of Vitamin T in Germ-Free Animal Tissues, Dr. Fraenkel of the University of Illinois.

Chick tissues were shipped to Dr. Fraenkel for the analysis of carnotine. He reported via letter that this material (Vitamin T) was present in germ-free chick muscle.

D. Future:

The work of the next six months will build upon the work done to date. We expect to complete the evaluation of the merry-go-round cage, the writing for the third LOBUND Report, the analytical work on the germ-free rat survey and the initial phase of the antibiotic problem.

There are many needs in the biochemistry laboratory for a more efficient operation. The lack of the following has made our work less complete:

1. Adequate standardization of stock animals (particularly more space needed).

2. Centrifuge, standard type or refrigerated.

3. Respirometer, Warburg.

4. Reading device for paper chromatographic and paper electrophoretic studies.

5. More space in the diet laboratory.

A request has been submitted to R. Ervin for the above apparatus if in Navy stocks or available elsewhere.

IV. BACTERIOLOGY AND SEROLOGY

FROM: M. Wagner, Chief Bacteriologist
(With the assistance of J. D'Agostino, A. DeLeva, B. McClain and M. Osterhout).

TO: J. A. Reyniers, Director

SUBJECT: LOBUND-ONR Report, 1 July 1952 - 31 December 1952

DATE: 31 January 1953

A. Bacteriological Testing of Germ-Free Cages

The function of the bacteriology laboratory continues as a service to various LOBUND projects being run in germ-free type apparatus.

B. Bacteriological Aspects of LOBUND X-Ray Radiation Studies

Bacteriological examination of rats which have died after X-ray radiation have been made. The data is recorded in the addendum to this progress report: "LOBUND Study on Comparative Effects on Total Body Radiation II".

C. Post-Mortem Bacterial Invasion into the Peritoneal Cavity of the Conventional Albino Rat.

A preliminary study has been made to determine the approximate time at which post-mortem bacterial invasion takes place into the peritoneal cavity of the conventional non-irradiated albino rat. The data is recorded in the addendum to this Progress Report: "LOBUND Study on Comparative Effects on Total Body Radiation II".

D. Intestinal Penetration by Bacteria and Bacterial Products (in collaboration with Physiology-Pathology Laboratory See Section V).

E. Dental Caries Project (Collaborative Project between LOBUND Institute and the Zoller Memorial Clinic, Univ. of Chicago).

The present phase of the dental caries project is to study the role of single-type bacteria in the caries process. Germfree animals are purposely inoculated orally with a viable pure culture of the test organism and maintained as a mono-contaminated group on a diet, which in the conventional laboratory rat produces a high incidence of dental caries.

Experiment 39D1-9 involves the use of the lactobacillus (Lactobacillus #465) in such a study. The outline of this experiment was presented in the progress report covering the period January to June, 1952: pgs. 20-21.

Briefly, 12 germfree rats were divided into three groups of 4 rats each.

Group A: Four rats maintained germfree throughout the experimental period on cariogenic diet L-128 + 5% dextrose water for 150 days.

Group AI: Four rats maintained germfree 48 days and then inoculated orally with viable Lactobacillus #465. Maintained as mono-contaminated animals in germfree-type equipment for 150 day experimental period on cariogenic diet L-128 + 5% dextrose water.

Group AIB: Same as group AI except that after oral inoculation, the four rats were brought out into the conventional animal colony environment and maintained on the cariogenic diet L-128 + 5% dextrose water for 150 days.

Group B: Set up independently using the conventional laboratory rat fed the autoclaved cariogenic diet L-128 + 5% dextrose water. This group was used as a dietary control to demonstrate that in the animal colony environment the conventional rats will develop dental caries in a 150 day period on the diet.

Three objectives were proposed:

1. Examination of the molar teeth for caries evaluation.
2. Study qualitatively and quantitatively the lactobacilli in the oral cavity of the rats constituting groups AI, AIB and B and compare to total bacterial count.
3. Observe survival of the specific test organism, Lactobacillus #465 in group AIB brought to the "outside conventional environment".

Groups A, AI and AIB have been sacrificed and the heads sent to the Zoller Memorial Clinic for caries evaluation. Group B is still on expetiment at this writing.

Gross examination of the molar teeth has revealed:

Group A - no gross lesions evident.
Group AI - no gross lesions evident.
Group AIB - gross lesions present.

Preparation of sections for microscopic examination of the molar teeth is still underway at Zoller Clinic. The final gross and microscopic evaluation for all groups will be available for the next Progress Report.

The oral bacterial counts run on the rats from Groups AI, AIB and B are recorded in the accompanying table and graph. It is to be noted that since Group AI is the group harboring only Lactobacillus #465, the data given for Total Count, Total Lactobacillus Count (all types) and Lactobacillus #465 Count are the same for this group.

Bacterial Counts from the Oral Cavity of Rats on the Dental Caries Project 39DI-9

Bacteria	Rat Group	No. Rats	No. Cultures	Bacteria Per Gram Sample		
				Minimum	Maximum	Average
Total Count on Blood Agar (anaerobic)	AI	4	24	9.50×10^2	1.50×10^6	1.52×10^5
	AIB	4	42	2.72×10^6	8.63×10^8	1.56×10^8
	B*	4	28	2.74×10^7	1.98×10^9	3.80×10^8
Total Lactobacilli (all types)	AI	4	24	9.50×10^2	1.50×10^6	1.52×10^5
	AIB	4	44	5.0×10^4	2.85×10^7	2.57×10^6
	B*	4	28	1.91×10^5	3.41×10^7	4.07×10^6
Lactobacillus #465 only	AI	4	24	9.50×10^2	1.50×10^6	1.52×10^5
	AIB	4	44	0**	1.13×10^7	1.14×10^5
	B*	4	28	0	0	0

* Additional data from Group B still forthcoming.

** Lactobacillus #465 was not detected in 9 of 44 cultures run in this group (20.4% negative cultures). Negative cultures obtained in each of the four rats comprising the group.

The data in the above table is shown graphically (fig. 1). The bars express the minimum-maximum range of the counts observed. The dotted line in each bar represents the average count for the group.

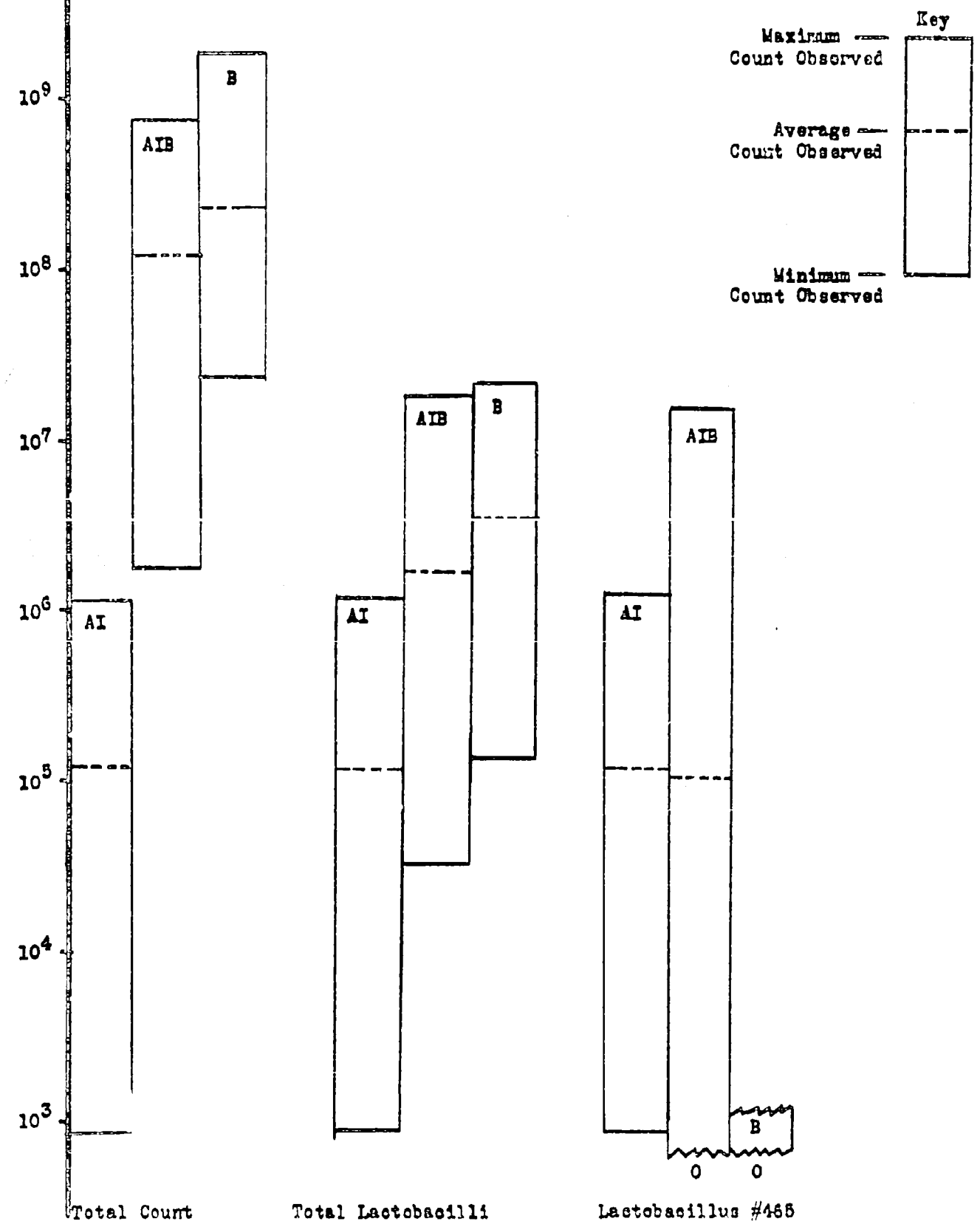
There was considerable variation in the successive counts made on individual animals as well as among the animals within each group. No one rat showed consistant high or low counts.

The average total counts for the AIB and B rats (housed in conventional animal room) were respectively 1000 and 2500 times greater than the average count for group AI (mono-contaminated group). There was no overlap in maximum AI total count and minimum AIB or B, total counts.

Regarding total lactobacilli, groups AIB and B exceed the AI average count by 17X and 26X respectively. There was an overlap between the maximum AI count and minimum AIB and B counts.

The Lactobacillus #465 data involves the specific organism orally inoculated into groups AI and AIB. This organism persisted in the AIB group after the rats had been brought out into the conventional animal colony environment. Variation in Lactobacillus #465 count was much greater in the AIB group. In 9 out of 44 cultures run, the L-#465 was not detected. At other times, counts higher than the maximum group AI count were encountered. On an overall average, groups AI and AIB were practically identical.

Figure 1


Key
Maximum Count Observed
Average Count Observed
Minimum Count Observed
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
AI
AIB
B
0
0
Total Count
Total Lactobacilli
Lactobacillus #465

Group B rats were not orally inoculated with Lactobacillus #465 and the organism has not been detected in this group to date.

An experiment similar to the one described above is currently being run at LOBUND. (index 39D2-1) However, in this case, Streptococcus liquefaciens #539 has been substituted for the lactobacillus as the mono-flora in group AI.

The rationale of the "lactobacillus" experiment has been the attempts to produce dental caries in a bacteriologically controlled environment, using a single type, highly acidogenic lactobacillus as the potential etiological agent. This comprises one test of the theory regarding the role of bacterial acidogenesis in the caries process.

The current experiment is set up on the same basis, but employs a strongly proteolytic organism, Streptococcus liquefaciens as a test of the proteolytic theory of cariogenesis. This organism is also acidogenic, although not to the extent exhibited by lactobacilli. Results from this experiment will be forthcoming in the next progress report.

F. Intestinal Microflora Studies: (Preliminary)

Bacterial counts were made from the contents of various segments of the gastro-intestinal tract taken from:

(1) Rats maintained in germ-free type apparatus with a single type bacterial contaminant. (Monoflora)

(2) Rats maintained under conventional animal room conditions (Polyflora).

The objective was to ascertain whether bacteria, existing as a monoflora in the intestinal tract of the rat, attain numbers comparable to the numbers reported as "total count" in conventionally reared animals harboring many different types of microorganisms.

The following preliminary data was obtained from 3 pairs of rats all fed diet L-128. The first and third group also received 5% dextrose water ad lib whereas the middle group was fed distilled water.

Bacteria per Gram (Dry Wgt) Gastro-Intestinal Contents

Segment	Bacterial Groupings					
	Lactobacillus #465 Monoflora (Code: 39D1-9 AI)		Bacillus cereus Monoflora (Code:53H9		Conventional Polyflora (Code 39D1-9AIB)	
	Rat #16	Rat #21	Rat #9	Rat #10	Rat #23	Rat #25
Stomach	1.59×10^5	1.69×10^4	1.61×10^5	5.72×10^4	3.70×10^8	4.53×10^9
Mid-intestine	1.22×10^5	1.26×10^5	5.13×10^4	2.09×10^6	3.26×10^8	6.54×10^8
Cecum	2.48×10^9	3.80×10^9	5.69×10^5	7.58×10^7	1.12×10^{10}	1.92×10^{10}
Colon	1.77×10^9	6.21×10^9	9.23×10^5	1.67×10^6	2.37×10^{10}	2.30×10^{10}

The stomach and mid-intestine of the two monoflora groups were lower than that of the conventional group.

The cecum and colon counts in the Lactobacillus monoflora group were considerably greater than counts in the upper segments; (i.e. minimum difference approximately 11,000 X; maximum difference approximately 360,000 X). This wide spread between the upper and lower segments was not seen in the conventional group where the minimum-maximum spread was only approximately 2X to approximately 70X.

The Bacillus Cereus monoflora group did not show too great a difference in upper vs. lower intestinal segments. However, the cecum and colon counts were considerably lower than those in the conventional group. This lower count may be due to the fact that this group was not fed dextrose water.

G. Universal Serologic Reaction with Lipid Antigen

Introductory remarks and data regarding the "universal serologic reaction" in germ-free chickens were presented in the Progress Report submitted for July-December 1951. At that time, there seemed to be a qualitative difference between germ-free and conventional chickens in reactions run with NaCl diluents at the 0 and 0.15% concentration, i.e. germ-free chicks failed to react in these lower NaCl concentrations while the conventional animals did.

Observations of several more chickens for each category has shown that these reactions are not clear cut (see figures 2 & 3). Thus, although 5 out of 6 germ-free cases were negative at the 0 and 0.15 NaCl concentrations in the germ-free (20-31 day age group) 1 case did react at these concentrations.

Universal Serologic Reaction
Distribution of Titers in Germ-Free vs. Conventional Chickens
(Age 20 - 31 Days)

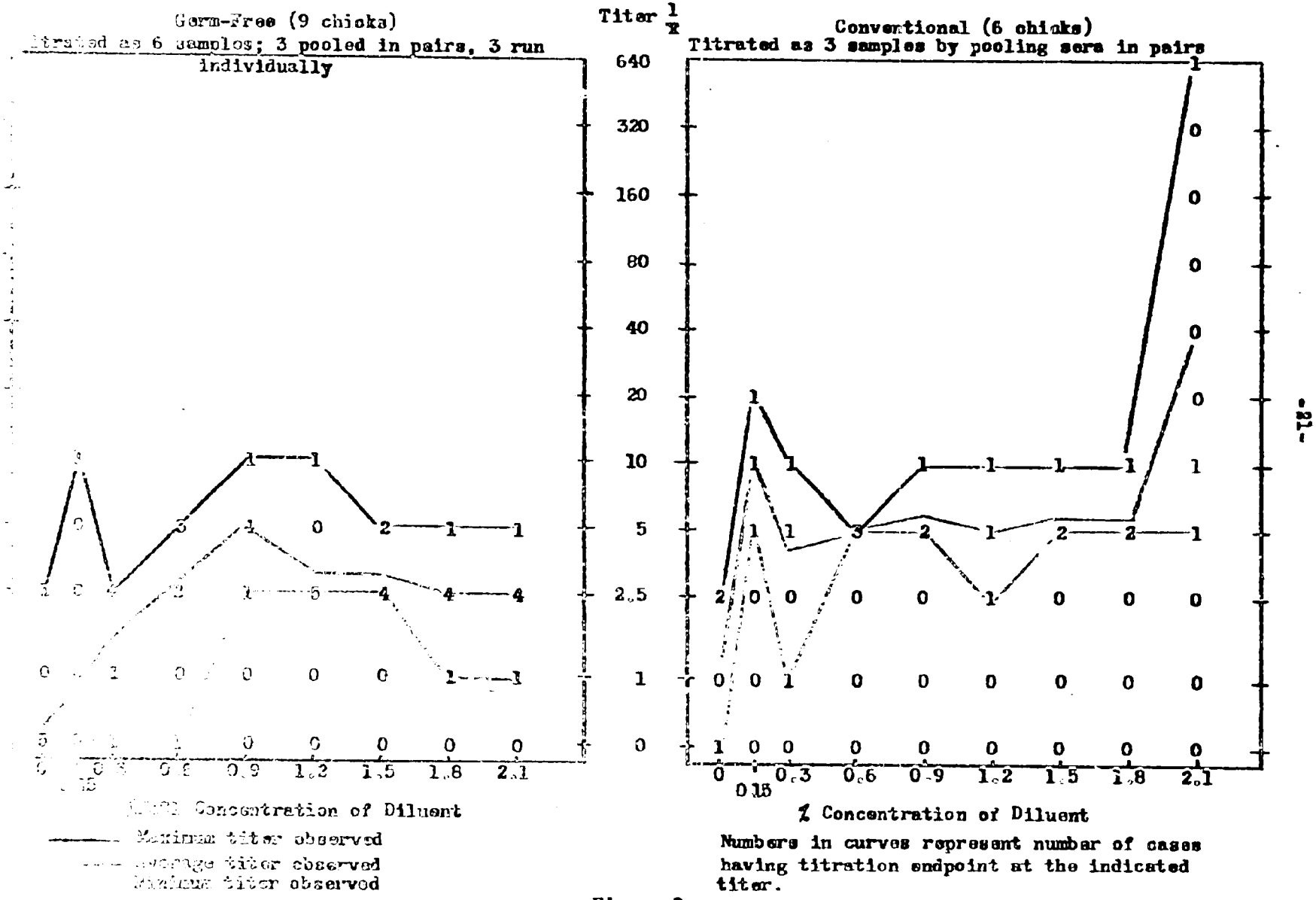


Figure 2

Universal Serologic Reaction
Distribution of Titers in Germ-Free vs. Conventional Chickens
(Ages 10 - 90 Days)

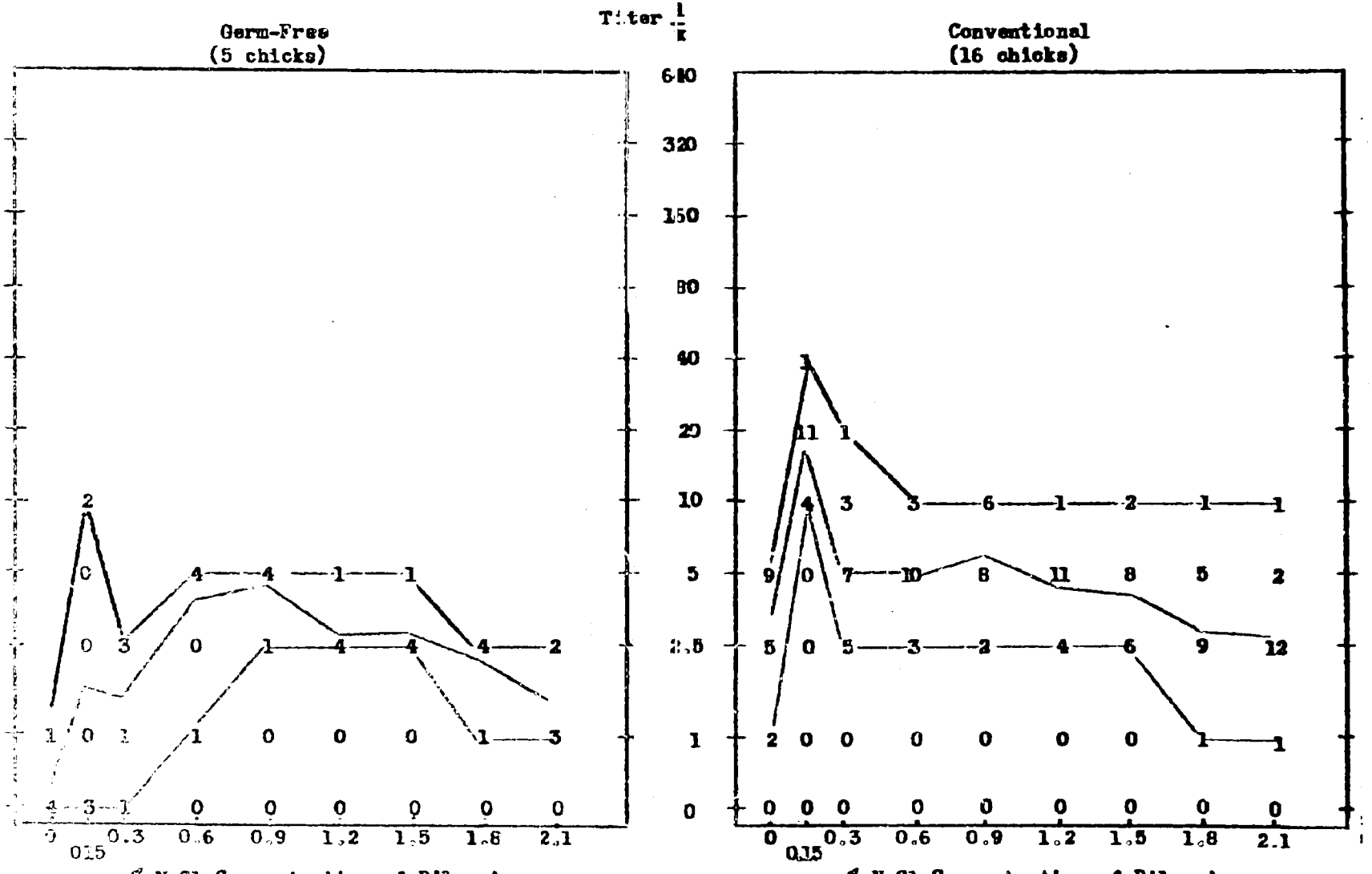


Figure 3

Similarly at the 60-90 day age level, 4 out of 5 germ-free cases failed to react at the 0 NaCl concentration whereas 1 of the 5 did react. These apparently aberrant positive reactions negate the uniformity of a negative zone in the serologic pattern.

However, from the quantitative standpoint at these lower NaCl levels, the average curve for germ-free serum at the ONaCl level is still very low even if these aberrant values are included. The conventional chickens show a distinct peak at the 0.15% NaCl test. This peak is not seen in the germ-free average curve. In general, the titers for germ-free chicken sera tend to be lower than serum taken from conventional birds.

It is also interesting to note that serum from 48 chicks at 0 - 1 day of age (run as 4 trials of 12 pooled sera each) failed to react at all NaCl concentrations from 0 to 2.1%.

H. Antibody Response in Germ-Free and Conventional Chickens to Parenterally Injected Antigens

In previous reports, the so-called natural antibody picture for germ-free and conventional chickens has been described. Currently, an experiment is underway to determine the antibody production potential of germfree vs. conventional chicks when parenterally stimulated with bacterial or foreign serum antigens. Results will be available for the next report.

V. PHYSIOLOGY AND PATHOLOGY

FROM: H. A. Gordon, Chief Physiologist (with the assistance of W. Scruggs and P. Wolfe. In collaboration with B. Phillips (NIH) who is in residence at LOBUND Institute)

TO: J. A. Reyniers, Director

SUBJECT: LOBUND-ONR Report, 1 July 1952 - 31 December 1952

DATE: 31 January 1953

The basic responsibility of LOBUND Institute is the performance of comparative studies between gnotobiotic* and conventional animals. Too early deviation from such survey type of work into specialized experimental fields has shown its undesirable effects through all history of germ-free research: insufficient knowledge of the various aspects of gnotobiosis has and may often lead to the improper evaluation of experiments where this form of life has been used as the test system. Thus we are convinced that, e.g., the germ-free animal when used in premature applied experimentation can have no lasting value among the tools of biological investigation. Therefore the objective of this laboratory is to perform anatomical, histological and physiological surveys in various gnotobiotic, mammalian and avian forms to supply sufficient comparative data. It is also felt that these should be complemented by a systematic study of the organisms' response to pathological stress. This is well within the LOBUND directives as set out in the original program.

The compilation of data in the past years followed this pattern as closely as it was permitted by the available animal material. As a result of this effort we have today a fairly reliable anatomical study of the germ-free chicken; a somewhat sketchy anatomical survey of the germ-free rat and a histological survey of the chicken; numerous spot checks in the histology of the rat as well as in the pathological responses of both germ-free and contaminated chickens and rats. Until the beginning of the period covered by this report we had not run any physiological tests on germ-free or control-contaminated animals.

In charting our future course, the original concept calls first for the completion of the morphological surveys. However, in this respect certain difficulties arose. The quantitative anatomical survey with the concomitant statistical evaluation must rely at least on tens of animals reared under identical environmental, dietetic, etc., conditions in order to obtain significance. This was made possible in the case of the Bantam chickens which finally permitted the completion of the anatomical survey of one germ-free form.

In respect to the rat it was originally hoped that the heat-catastrophe suffered in LOBUND's colony in 1951 would supply sufficient acceptable animal material for a quantitative anatomical survey. Unfortunately, the conditions of heat-killing as they occurred at that time influenced the dead animals in a rather adverse way, dimming the possibility to use them as the sole material in the survey. The main trouble

* The word "gnotobiosis" is used to define all forms of life maintained germ-free or in conditions of controlled contamination.

with this group seems to stem from the lack of uniform exsanguination which is of greatest importance in the analysis of organ weights. On the other hand, the isolated live germ-free rats of different dietetic, etc., background which trickled into this laboratory during the past years were simply insufficient to fill the various categories with the proper number of participants. Actually, we would do a disservice to our own germ-free animal material, if we would try to draw the "anatomical baseline" for the germ-free rat with the help of few heterogenous animals. Thus the completion of this survey will in all probability depend on the expansion of our production facilities.

The histological survey of both chickens and rats, though unfinished, is well under way. It follows from the nature of this kind of work that only fewer animals and ample time for microscopic observation are needed for this type of work.

These being the conditions in our laboratory at the beginning of the recent school year, we were faced by some pressing questions. As mentioned before, the completion of the morphological survey seemed to have the logical precedence. At the same time, however, chiefly because of the relative lack of experimental animals, a certain amount of time was left unused in our hands which we felt should and can be used for research.

In order to bridge this conflict we have begun to develop and refine some methods which promise to be advantageous in the pursuit of the future physiological survey. This decision was only strengthened by the lack of physiological information about our gnotobiotic animals which made itself felt rather intensely during the past years. Simultaneously, however, it was understood, that the completion of the morphological survey will maintain its first priority in our laboratory schedule.

Contributions to the problem of hand rearing mammals seemed to be a secondary, but none the less important task of this section. Therefore, additional experiments were performed in the physiology of intestinal absorption of the caesarian-born baby rat, during the first hours of life.

The experimental follow-up of some LOBUND commitments to various agencies should be also mentioned. Experiments run in collaboration with our AEC Advisory Board as well as with the Laboratory of Tropical Disease of the National Microbiological Institute are described in attached reports.

In the closing chapter a brief report is made of this lab's activity as a pathology service unit to LOBUND's germ-free animal production division.

In a summary the total number of animals processed in this laboratory during the past half year was as follows: 743 rats, 49 chickens, 74 guinea pigs.

A. Survey of Gnotobiotic Animals:

In the following paragraphs an attempt will be made to present a trend of thinking, a few technical approaches together with preliminary results which aim to explain in physiological terms the morphological findings hitherto made in germ-free and other gnotobiotic animals.

As mentioned in previous reports, morphological differences exist between germ-free and conventional animals in respect of (1) organs of systems which come in direct contact with the outside world (tegumentary, respiratory and digestive) and (2) in the reticuloendothelial system which at least in the conventional animal constitutes the cellular defense mechanism of the body. In both instances the germ-free animal seems to have less or less well trained tissue available.

In the selection of a proper chronological sequence for the proposed physiological study, it appeared that the digestive system should have precedence over the others because of its overall important role in the host contaminant relationship. Among its functions it was tentatively decided to investigate first: the absorption and secretion of foodstuffs and soluble bacterial products from and into the intestinal canal; penetration of bacteria from the intestinal canal into the host. Both projects were planned to be studied in cooperation with LOBUND's biochemistry and bacteriology laboratories.

In respect of the study of the reticuloendothelial system it was felt for a long time that the customary methods of reading results (hematology and histology) are in great need of refinement in the observation. Reticuloendothelial cell production and utilization (breakup) in various sites of the organism seem to require further elucidation, or more precisely, need was felt for preparing leukocytic balance sheets at the organ level (as Yoffey and others have done it for the lymphocyte in the organism as a whole). It was also suggested that this work should be paralleled by serological observations in LOBUND's serology laboratory.

B. Techniques:

Methodologically, it appeared that the initial phases of both projects (intestinal penetration and leukocytic balance) should be studied in an acute experiment. The reduction of the period of observation to a few hours after the animal's removal from the germ-free unit together with the reduction in the site, where the observation is made in a single organ or in a part of an organ (such as one portion of the intestine) tends to reduce variables and makes observations more specific. Besides, it also permits to perform such delicate operations under less of a handicap.

Intestinal absorption and secretion is studied with the "isolated loop" technique in the "retrieving" type experiment (a loop at the desired height, in the length of 1 - 2" is ligatured at both ends maintaining proper blood circulation, Photo 1: the substance whose passage will be studied is introduced in known amounts into (1) the loop (absorption), or (2) into general circulation (secretion); after the lapse of known time, the substance is retrieved from the loop). This type of information is supplemented in the present experiments by simultaneous determinations of the substance in question in (a) mesenterial venous blood draining the loop, (b) chyle from the mesenterial lymphatic trunks, (c) mixed venous blood from the cava system, (d) arterial blood, Bacterial penetration is studied with the help of the same method under sterile cautelae, omitting only the retrieving.

In respect of the positive or negative organ contribution to the circulating reticuloendothelial cell picture, it was decided to perform initially a survey on the leukocyte-concentration (differentiated into granulocytes, lymphocytes, etc.) in the venous blood of various organs, together with similar counts in the arterial blood. The necessity of simultaneous determination of blood flow measurements was clearly visualized at the present time, but omitted because of technical difficulties. Proper blood and lymph samples were obtained by the use of polyethylene tubing mounted on narrow gauge syringe needles. Cannulation was performed manually under loupe-magnification.

C. Preliminary Results:

1. Absorption and Secretion Studies of Foodstuffs (in collaboration with Biochemistry)

A mixture of B vitamins was used for this purpose in a preliminary experiment. The site of absorption or penetration was the lower small intestine. The animal categories studied were: germ-free, di-contaminated, conventional rats. The analytical results have not been evaluated yet.

2. Penetration Studies of Bacteria and Bacterial Products (in collaboration with Bacteriology and Biochemistry)

Pilot experiments were run to study the absorption of indole and the penetration of Salmonella Typhimurium from the isolated intestinal loop. No results are available at this writing.

3. Leukocyte Concentration Studies in the Venous Blood of Various Organs

The changes resulting in the leukocyte concentration of the blood during its passage from artery to vein in various organs are shown in one germ-free, one dicontaminated and one conventional rat (Figures 1, 2 and 3). The increase or decrease of the leukocytic elements in the venous blood are expressed in absolute values on both sides of the abscissae, the latter representing the arterial mean. The values are tentatively corrected for the arterio venous concentration factor (resulting from the filtration of lymph) as well as for various exposure effects which are the consequences of the experimental procedure (repeated blood-letting, etc.). The values presented in the bar graphs are the means of repeated observations; the difference in successive counts of the same sample was usually less than 10%. The results, as far as it is permissible to speak of such at the present time, can be read at a glance from the figures.

D. Study on Comparative Effects of Total Body Radiation:

See attached report which follows Section VIII.

E. Amebiasis Investigations in Germ-Free Guinea Pigs (with Dr. Wright, Dr. Rees and B. Phillips of the Microbiological Institute of NIH).

See progress report prepared by B. Phillips in Section VII.

F. Service Functions:

During the period covered by this report (from August through December, 1952) animals of a wide range of ages and both sexes from the

Figure 1

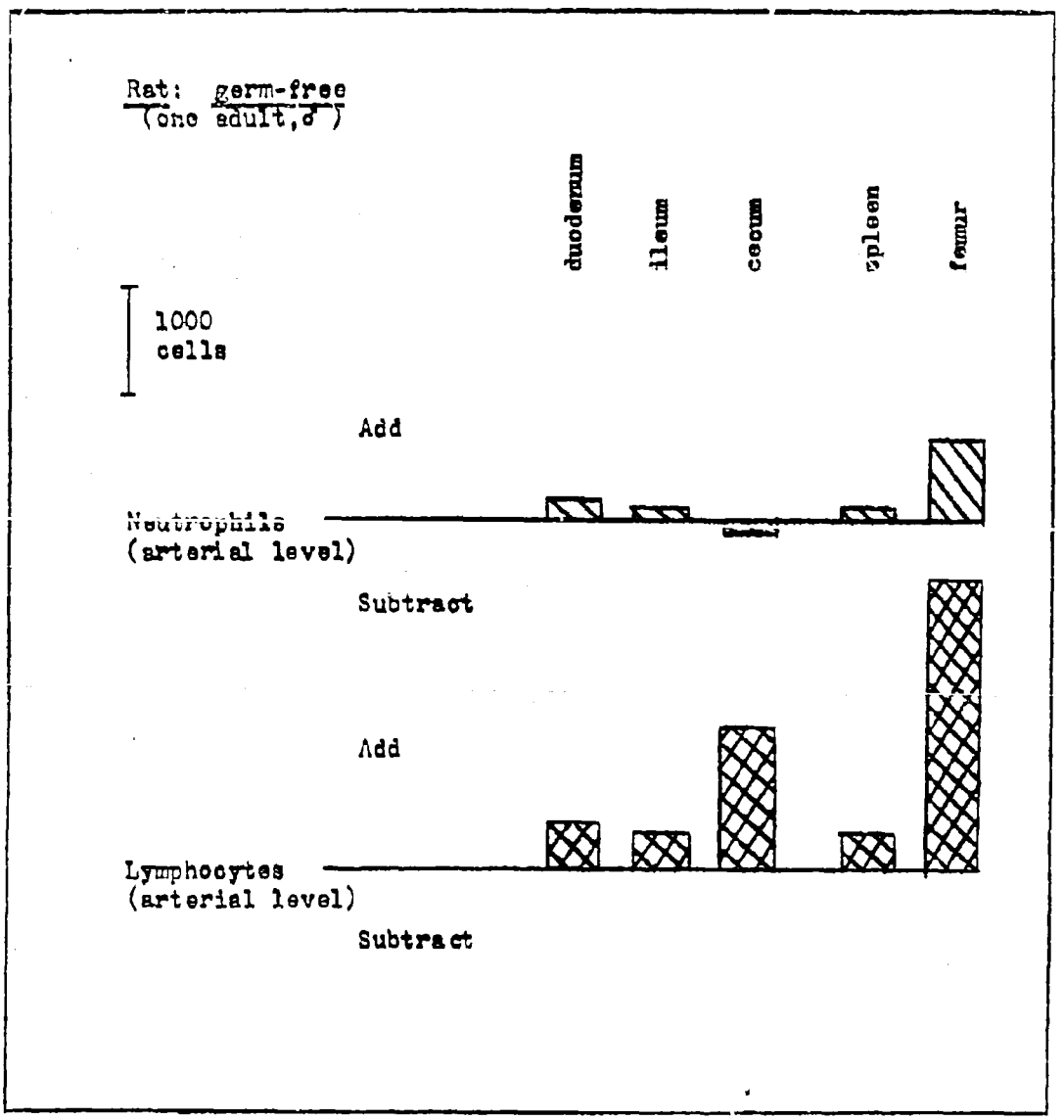

Rat: germ-free
(one adult, ♂)
duodenum
ileum
cecum
spleen
femur
1000
cells
Add
Neutrophils
(arterial level)
Subtract
Add
Lymphocytes
(arterial level)
Subtract

Figure 2

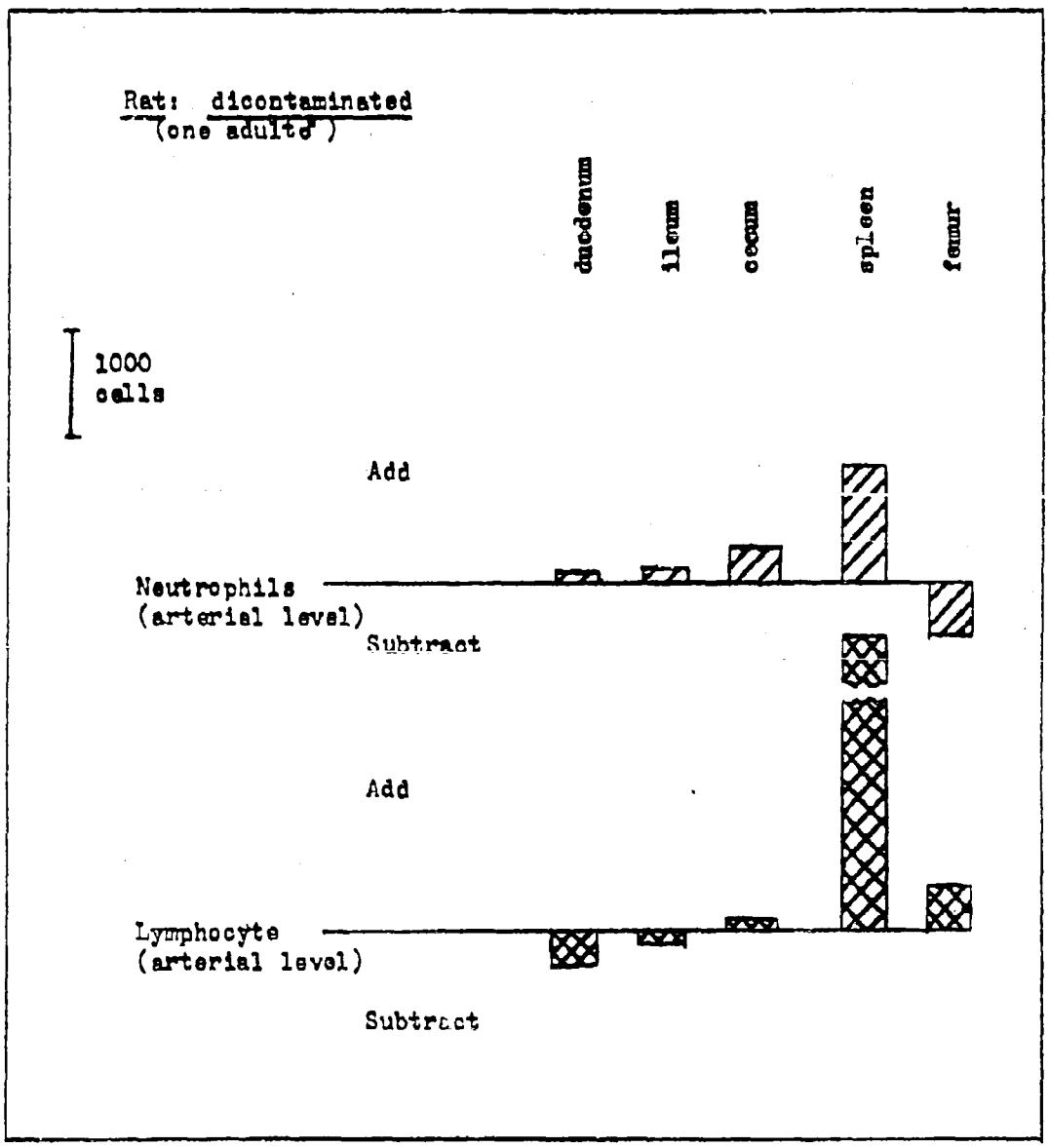
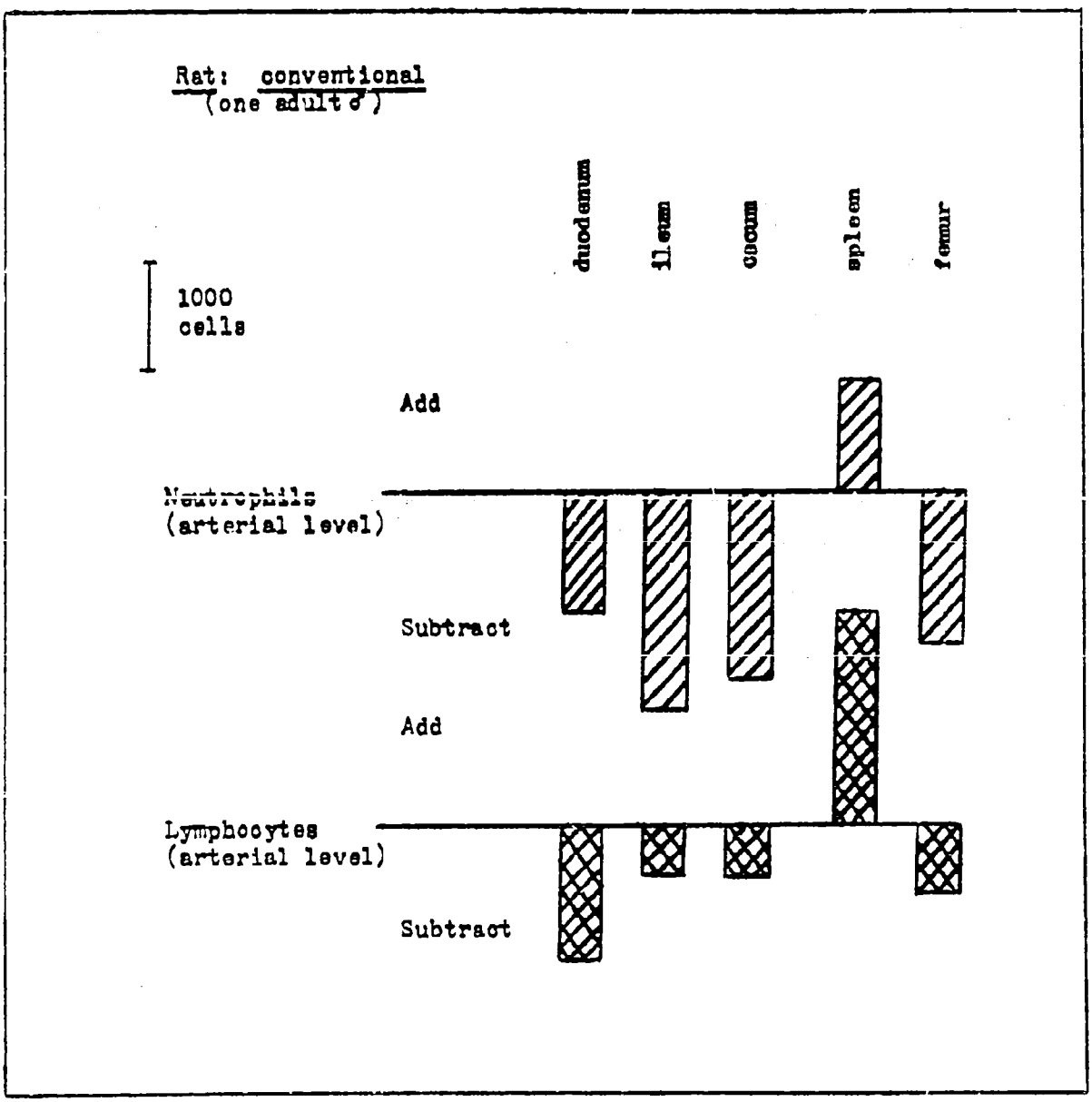

Rat: dicontaminated
(one adult)
duodenum
ileum
cecum
spleen
femur
1000
cells
Add
Neutrophils
(arterial level)
Subtract
Add
Lymphocyte
(arterial level)
Subtract

Figure 3


Rat: conventional
(one adult ♂)
duodenum
ileum
cecum
spleen
femur
1000
cells
Add
Neutrophils
(arterial level)
Subtract
Add
Lymphocytes
(arterial level)
Subtract

germ-free stock colony of rats died at a rate of from 5 to 10 per month; additionally, several litters of suckling, Caesarian born rats were lost. As a service function, personnel of this laboratory performed autopsies on most of these animals.

It was found that aside from an occasional mature animal which died because of sequelae of volvuli usually involving the distal 1 to 2 centimeters of the ileum and the proximal 1 to 2 centimeters of the colon, most of these animals succumbed of asphyxiation. This asphyxia could neither be demonstrated to be due to mechanical obstruction (e.g., strangulation, smothering, or drowning) of the respiratory passages nor have we been able to demonstrate that it was due to conditions giving rise to hypoxia.

Photo 2 is of an animal which died under these, so far inexplicable circumstances. The probe indicates all that could be found of lung tissue and this was completely hepatized, filling approximately 1/5 the volume ordinarily occupied by normal lungs. While the lungs of other animals with this syndrome were not usually so profoundly changed, they differed only in degree, not in kind. The outstanding gross pathological finding common to every animal with the syndrome can be characterized as being a pronounced pulmonary hyperemia uniformly distributed throughout both lungs with irregularly spaced areas of hepatized tissue, (on section the cut surface of the lungs showed these hepatized areas to extend wedge-wise below the surface towards the hilum), to about 1 sq. cm. Occasionally, the free margins of the lungs are found tightly adherent to the parietal pleura and in other instances varying quantities of a non-purulent, moderately viscous, sero-sanguinous fluid is found in the pleural cavity. Histopathological examination of the lungs revealed the underlying cause of the gross findings to be a hemorrhagic bronchopneumonia.

This laboratory is continuing its efforts to determine whether the causative agent is physical in character or whether genetic factors are responsible. In addition, the staff of the Virology Laboratory has initiated experiments designed to test the possibility that non-physical factors (other than those of genetic origin) may be operating to give rise to this lung syndrome. Their efforts concerning this are described in Section VI of this report.

Service function rendered in response to a request from an investigator other than a member of the LOBUND Research staff. Description of a technique. (With the collaboration of A. N. Lorenc, Supervisor of the LOBUND Animal Colony).

Most workers using animals for experimental purposes agree that it is virtually impossible to obtain them free of disease from any of the commercial suppliers of animals. This constitutes a problem of such magnitude, for example, that it occupied most of the time of the Animal Care Panel* held on December 3 and 4, 1952.

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* 3rd Annual Animal Care Panel, University of Illinois College of Medicine, December 3 and 4, 1952; attended by one member of this laboratory and A. N. Lorenc.

Photo No. 1 - An isolated loop from the lower portion of the rat's small intestine with cannulae in situ.

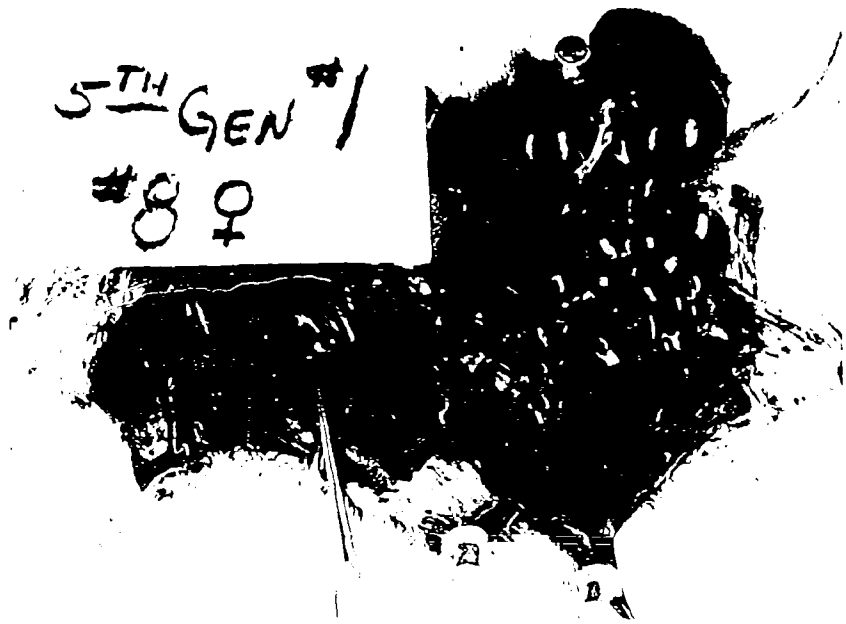

Photo No. 2 - The lung syndrome observed in some germ-free rats. Probe indicates all that could be found of lung tissue.

In August, 1952, the research staff of LOBUND was approached by Mr. Evan Holtzman of Holtzman-Rolfsmeyer Co., Madison, Wisconsin with an appeal to give him help in establishing a disease-free colony of rats of the Holtzman strain. He was aware that we were in a unique position to help him since our own staff recognized several years ago that clean, healthy stock animals (which are indispensable in the building of a germ-free animal colony of sufficient size to perpetuate itself) were generally unavailable. Consequently, we developed our own disease-free colony of conventional rats, stocking it originally with the only surely disease-free progenitors known, namely, germ-free animals. Our method of maintaining a disease-free rat colony has been reported in previous progress reports.

Mr. Holtzman was asked to deliver to us several of his pregnant dams with gestation periods timed such that parturition should occur simultaneously with that of similarly timed disease-free LOBUND strain rats. On September 26, 1952 the procedure was initiated and carried out such that for each LOBUND dam casting a litter, a Caesarian section was done on a Holtzman dam. The LOBUND litter was counted and removed from its dam after which the same number of Caesarian born Holtzman babies were substituted for it, using techniques which insure that a foster mother will nurse and care for the substitute litter. During this procedure the Holtzman and LOBUND females were kept properly isolated from each other and the Caesarian procedures were done using sufficiently aseptic methods to prevent contamination of the young by the adult Holtzman rats. Thirty-four infant Holtzman rats were weaned and delivered to Mr. Holtzman as the nucleus of a disease-free colony of his strain of animals.

VI. VIROLOGY*

FROM: J. F. Reback, Virologist
(With the assistance of M. Sacksteder).

TO: J. A. Reyniers, Director

SUBJECT: LOBUND-ONR Report, 1 July 1952 - 31 December 1952

DATE: 31 January 1953

During the past six months the attention of the Virus Laboratory has been directed to the following projects:

A. Studies on the action of the viruses of lymphomatosis, Theiler's encephalomyelitis, and human infectious hepatitis on germfree chickens.

B. The development of methods for the detection of latent or symbiotic viruses in germfree animals. (1) Studies on possible virus-microbe associations. (2) Attempt at X-ray activation of latent viral agents in germfree chicks. (3) Attempt at X-ray activation of symbiotic viral agents in germfree chicks. (4) Investigation of spontaneous disease manifestations in germfree chicks, not attributable to bacterial infection (e.g. neurogenic disease of germfree chicks, lung condition in germfree rats).

C. Attempt at filtrate passage of methylcholanthrene-induced tumors in germfree chickens.

No complete report for any one of these studies can be given as yet. Hence, the following sections are of the nature of progress "briefs", indicative only of some of the ground covered so far, while allowing the probability that later results, in most of the projects, will make complete discussion practicable at some future time.

A. Action of Viruses in Germfree Chickens

1. Lymphomatosis

Our studies are currently limited to two phases: (1) study of the action of the filtrable agent in susceptible Germfree chicks (Regional Poultry stock); and (2) observations for egg transmission of the causative agent in germfree chicks.

* The ~~principle~~ *principal* support for these studies in virology is received from the Damon Runyon Memorial Fund.

Of six experiments designed for attempt at filtrate passage of the agent of lymphomatosis under germfree conditions, four have been discontinued because of contamination or of exceptionally poor hatch from the Regional Poultry Research Laboratory (East Lansing, Michigan) egg stock. Two of the germfree experiments with this agent are progressing satisfactorily.

In a seventh experiment, eggs from infected stock have been hatched in the germfree system, and it is planned to hold the chicks for long term observation, as a direct inquiry into the problem of egg transmissibility of lymphomatosis.

Final report on all phases of this work will be made at a later date.

2. Theiler's Mouse Encephalomyelitis

The object of the series of studies, initiated by the experiment to be reviewed briefly here, is to test survival or persistence of a viral agent in an unnatural host. At the same time, observations of the host for effects possibly due to the presence of the viral agent are not neglected.

Five intracerebral passages through mice were made of the FA strain of Theiler's virus. The final pooled harvest, suspended in 1:1 glycerine-saline (V/V) , was partially purified by differential centrifugation. A 1:10 dilution of this preparation, inoculated intracerebrally (0.02 ml each), killed 100 percent of mice in six days; a 10^{-3} dilution killed 100 percent of mice in nine days.

The partially purified, glycerinated virus preparation was diluted 1:5 with saline and then passed through a coarse frittered glass filter (4Q4 pore size), after which an ultrafiltrate of this material was prepared. The ultrafiltrate showed an LD_{50} (IC in mice) at a dilution of 1:150.

The undiluted ultrafiltrate was inoculated into five 3½-day germfree chicks (White Leghorns, Hi-Vue Farms stock), as follows:

Chick No.	Inoculation Data
261 and 267	IM (pectoral), 0.5 ml each.
262 and 268	IC, 0.07 ml each.
269	IV, 0.5 ml

None of the chickens showed any ill effects whatsoever following inoculation. The germfree state (except for the virus) was preserved throughout the period of observation. Sacrifice was made at different times, as follows:

Chicken No.	Period of Observation After Inoculation
267	35 Days
268	43 "
269	49 "
262	62 "
261	71 "

In each instance, serum, brain, and intestinal contents were taken for tests in mice. The brain tissues and intestinal contents were prepared in separate 1:10 suspensions with saline (W/V). These were cleared by centrifugation at 2000 rpm for 10 minutes. The sera were diluted 1:10 with saline (V/V). The three preparations from each chicken, in the 1:10 dilutions, were inoculated intracerebrally (0.03 ml) into newly-weaned white mice.

The groups of mice were held under observation for six-week periods. In no instance was a definite sign of Theiler's disease observed.

3. Human Infectious Hepatitis

Our original objectives in this study may be given as: (1) to determine what avtivity, if any, the virus of human infectious hepatitis might show in GF chicks, and (2) to determine whether incidence might be abetted by sub-lethal X-ray preparation of the host. The early results proved encouraging, and it was decided to investigate fully the possible usefulness of the GF chicken as a laboratory host for the agent of this disease.

As of this date, seven GF experiments concerning this problem have been completed, an eighth is presently underway, and several further experiments are planned. It is hoped that a complete review covering this project may be presented in our next Semi-Annual ONR Report.

For the present, it may be said that we have observed illness and death in GF birds at periods of two to three weeks following inoculation with hepatitis serum ultrafiltrates. It would seem that this must be ascribed to some infectious agent, evidently of viral nature. The progressive development of symptoms and the prolonged moribund state would point to this. To classify the observed response as allergic in nature would appear questionable since any sort of allergic phenomenon, it is offered, would probably become manifest earlier and show, as well, a less leisurely sequence.

The principal factor of the syndrome would appear to be a pronounced gastro-intestinal disturbance, evidently centralized in the crop. Usually the crop appears bloated, containing either much foam or much fluid, or both. Marked catarrhal discharge occurs from the mouth. In this stage the birds give indication of extreme weakness, both in appearance and to handling. Thereafter the usual sequence is flaccid paralysis, muscular dystrophy, and finally the moribund state. Sacrifice and necropsy of such birds generally incriminates the crop and its contents, as already mentioned, usually with no signs of ill effect in other viscera, under either gross or microscopic examination. Considering the circumstances, it is to be observed that there is in these birds a singular absence of liver involvement.

We have attempted to characterize the responsible agent by recourse to serological, cytological, and cultural tests. The results have not proved overly gratifying (to be detailed in the future complete report).

Incidence in GF chickens, following inoculation, has proved erratic. While whole groups of GF birds have manifested the syndrome previously described, it is also true that entire groups have shown no response whatsoever. In our earlier, apparently "successful" work, the usual procedure was to inoculate intravenously. However, in a later experiment, attempt being made to determine optimal route of inoculation, it transpired that birds to which virus ultrafiltrate was administered orally or intramuscularly responded, while birds of the same GF group inoculated intravenously or intraperitoneally did not.

To date, incidence, when it occurs, has been observed only in 30-day or older GF chickens. Two groups of younger GF birds (8 and 9 days old at the time of inoculation) proved resistent.

No reaction and no recognizable pathology has thus far been obtained in inoculated conventional chickens. Some birds of such groups have also been X-ray irradiated. After inoculation, these "conventional" groups are isolated inside a GF "examination chamber," for protection of personnel.

We have used strains of infectious hepatitis virus from both the Akiba (Pennsylvania) and the New Lisbon(New Jersey) epidemics. Our evidence indicates successful transinoculation of an infectious agent from the Akiba material through three successive groups of GF chickens. An attempt at 4th passage of this agent in GF chickens is presently underway.

Our limited data would seem also to indicate that exposure of the host to X-ray radiation of sub-lethal dosage serves to shorten the incubation period with the infectious agent.

Phases of the problem currently under consideration include: (1) the attempt at 4th passage of the agent in GF chickens, (2) the question of an optimal route of inoculation, (3) the problem of the minimal infective dose, and (4) the design and conduct of a satisfactory neutralization test employing active GF chicken serum (or other tissue) against both pre-infection and convalescent human sera. The latter three phases are obviously intended to establish the identity or non-identity of the agent with the virus of human infectious hepatitis.

D. The Development of Methods for the Detection of Latent and Symbiotic Viruses in Germ-Free Animals.

A few remarks of a general nature, yet bearing on the rationale for this section, will be found in the Radiation Injury Report which follows Section VIII.

1. Studies on Possible Virus-Microbe Associations

Our investigation into the possibility of propagation or survival of different viruses when brought in contact with certain microbial cells is motivated by the theoretical potentialities of such virus-microbe systems toward the detection of latent or symbiotic viral agents in animal hosts.

Other workers have attempted to induce viral propagation by rather similar procedures, suggestive results being reported by Silber and Wostrouchowa (1932, 1933), Silber (1935) and Khurgina (1935)with growth of vaccinia virus on yeast; by Isabolinski, Lewzow, and Tschernjah (1935) with cultivation of vaccinia virus on fungi; by Bolin and Anderson (1947) with absorption of Mouse-Hamster (M-H) virus on cells of Micrococcus varians, possibly with metabolic involvement of the latter; and by others (references given by Silber). Essentially negative results in this direction have been reported by Amies (1934), Voet (1935), and Lenz (1937) in attempting to grow vaccinia on yeasts, and by Shaw and Dalldorf (1950) in attempting to correlate the presence of Theiler's mouse encephalomyelitis virus (TO strain) with changes in the intestinal flora (an in vivo study with mice).

We have thus far directed our attention, mainly, to four viral agents, viz. the viruses of Theiler's mouse encephalomyelitis, Rous Chicken sarcoma, Influenza PR8, and Kikuth's canary pox. Strains of pneumonia virus of mice (PVM) and Baker's feline pneumonitis virus, also tried, did not prove suitable. Methods of preservation and titration of these viruses have been tested and established under the conditions and facilities of this laboratory. Microorganisms used in conjunction with these agents are Saccharomyces cerevisiae Carlsbergensis, Rhodotorula rubrum, a pure British baker's yeast, Staphylococcus aureus, Escherichia coli, Bacillus subtilis, and Trypanosoma cruzi (Table I).

In general, our procedures conform to the method followed by Silber (1933). Virus material is introduced into young broth cultures of the microbial cells and the mixed entities (virus-microbe systems) are transferred to fresh broth at two to four day intervals, through varying numbers of transplants. Attempt to detect presence of virus in any particular transplant is made by an applicable, dependable method (Table I). i.e., serologically or by appropriate inoculation into mice, chicks, or embryonated eggs. Colorimetric and pH effects of the virus-microbe associations are sometimes followed. Inclusion bodies may be sought (especially with the viruses of canary pox and feline pneumonitis).

Other observations for viral effects have been made, for example: (1) effect of virus-inoculation of giant microbial stab colonies on agar, and (2) effect of presence of virus on individual microbial cells (this being determined by the study of living slide cultures and of stained preparations).

Table -I- VIRUS - MICROBE ASSOCIATIONS TESTED FOR PROPAGATION OR SURVIVAL OF THE VIRUS

VIRUS TESTED	MICROBE USED							Method for detection of the virus
	S.cerevisiae Carlsbergensis	R.rubrum	British bakers' yeast	S.aureus	E.coli	B.subtilis	T.cruzi	
THEILER'S	X	X	X	X	X	X		Intracerebral inoc. into newly-weaned mice.
ROUS CHICKEN SARCOMA	X	X	X	X	X	X		Inoc. into pectoral muscle of 30-day chicks
INFLUENZA A	X	X	X	X	X	X	X	Hemagglutination test
CANARY POX	X	X	X					Inoc. into chorio-allantoic membrane of 10-13 day embryos
FELINE PNEUMONITIS	X							Inoc. into yolk sac of 6-day embryos

"X" indicates tests havebeen conducted on designated virus-microbe system.

At one time or another in the course of our study, we have observed indicative results with the following virus-microbe combinations:

No.	Virus-Microbe Association	Observed Effect
1.	Influenza PR8 and S. aureus	Lysis.
2.	Theiler's virus and B. subtilis	Inhibition of sporulation.
3.	Rous sarcoma and S. cerevisiae	Prolongation of natural sedimentation period.
4.	Rous sarcoma and E. coli	Growth inhibition and/or lysis (?).

In no instance has it been possible to duplicate results in any consistant fashion.

Much of the work is being repeated, with certain refinement and greater emphasis on control. Recently attention has been directed at the metallic ion and the amino acid composition of the culture media, in the wake of seemingly rather valuable findings in published studies of bacteriophage adsorption, penetration, etc.

Thus, though we have already given considerable time and effort to this problem, the indeterminate nature of our results up to the present time compels us to regard the work as still in its preliminary stages. The investigation is continued on the assumption that the possibilities inherent in the method, as an indicator for the presence of inapparent animal viruses, have not been fully explored.

2. Attempt at X-ray Activation of Latent Viral Agents in Germ-Free Chicks.

See Radiation Injury Report which follows Section VIII

3. Attempt at X-ray Activation of Symbiotic Viral Agents in Germ-free Chicks.

See Radiation Injury Report which follows Section VIII

4. Investigation of Spontaneous Disease Manifestations in Germ-Free Chicks, not attributable to Bacterial Infection.

Neurogenic disease of GF chicks. "Jitters," a spontaneous neurogenic disease of very young GF chicks, has been under study in these laboratories for a number of years. This "natural" affliction of two or three day old germ-free chicks is characterized by tremors of definitive and epizootic aspect. Brain tissue from such birds has been harvested and is presently preserved in our CO_2 dry ice box until such time as germ-free facilities become available for an attempt at filtrate passage of the responsible agent into unaffected young GF chicks.

Lung condition in GF rats. A hemorrhagic bronchopneumonia-like condition has recently been observed in our GF rat colony. The condition seems to be channeled to most of the offspring of a few dams. Newborn to one year old rats (3rd and 4th GF generations) may be afflicted, but usually the period of manifestation is at 50 to 70 days.

Onset is sudden, mortality abrupt, death in such groups coming almost simultaneously to all members. Symptomology is poor, a marked nervousness being probably the most consistent diagnostic sign.

In our investigation of a possible virus cause, it is important that afflicted animals be detected while still living, to insure harvesting of the responsible agent in an active state. We have noticed that conventional mice and rats, which we have employed in transmission attempts and which have shown lung disease about 30 days after inoculation, also pass through a markedly nervous phase near what may be considered to be the end of the incubation period. This apparently important "clinical" sign should therefore prove useful in future effort. Doubtless, germ-free animals dead of the affliction for as much as one or two hours might well be utilized, autolysis with liberation of any viral elements possibly being rather advantageous than otherwise to our filtration procedures.

We have already made a number of attempts to transmit the disease to conventional animals by inoculation of lung homogenate from dead or moribund GF animals. In the test animals a very similar lung condition develops, when manifested at all, some 30 days after IV, IP, or IN inoculation, in both mice and rats. Since 50 to 70 day GF rats are predominantly afflicted, it appears indicatory henceforth that only animals from 0 to 25 days of age should be used for passage studies. We are presently using newly-weaned (3-4 week) mice and rats.

Unfortunately, our earlier results were complicated by the simultaneous occurrence of a lung disease epizootic in our conventional stock colonies. We have tried to offset this (1) by some measure of isolation of our experimental conventional mice and rats, and (2) by dividing offspring from the same dam into two groups: (a) inoculated with lung homogenate (or filtrate), and (b) untreated, but held under the same environmental conditions as group (a). Lines thus far not found to be naturally afflicted may also be used in these transmission studies. It is planned, when facilities permit, to conduct similar passage studies under GF conditions.

Continuous passage may of course alter the virulence and incubation period of the etiological (?) agent. Our attempts to induce the disease have yielded a fair percentage of strongly suggestive results with first passage, vis. 20 to 25 percent incidence. However, second and third passage trials thus far have proved less fruitful. Some few mice, succumbing early (one week) after inoculation with first passage material, have shown encephalitic symptoms.

C. Attempt at Filtrate Passage of Methylcholanthrene-Induced Tumors in Germ-Free Chickens

The object of this study is to attempt to produce tumor in GF chicks by inoculation of methylcholanthrene, and, if successful, to attempt to induce tumor in a second series of GF chicks by inoculation of cell-free material from the first tumor growths. Positive results in both parts of the study, if obtained, would point strongly to liberation of a transmissible, virus-like agent by this carcinogen.

To date, the present research group has made: (1) four attempts to establish tumor growth in GF chicks by inoculation of MChA, and (2) one attempt to incite tumor in GF chicks by inoculation of an ultrafiltrate prepared from MChA-induced tumor tissue.

Groups of about six GF birds at 5 to 9 days of age were used. In our two earlier experiments, we used White Leghorn chicks of Highview Farms stock (LaPaz, Indiana). In our later MChA-inoculation trials and in our attempt at tumor filtrate passage, we turned to White Leghorn chicks of Dembro stock (New Jersey), known to be responsive to MChA.

Inoculation in each instance was made intramuscularly (right breast) with 0.2 ml of a saturated, or near-saturated, solution of methylcholanthrene in benzene (ca. 1.9 percent MChA). At the same time, control GF chicks were inoculated IM with 0.2 ml of pure benzene alone.

Though contamination of the GF units occurred before completion of any of these experiments, it nevertheless seems evident from our results thus far that tumor growth may be induced in GF chicks with methylcholanthrene. One clear-cut case of tumor incidence under strictly Gf conditions has been observed, and it is quite likely that at least three other MChA-inoculated chicks showed incipient tumor or tumor-like response prior to contamination of the units. A number of birds, MChA-inoculated under GF conditions and thereafter maintained GF for 60 or 70 days, have shown incidence and development of tumor only after contamination, at periods ranging from three to five months after inoculation.

None of the birds inoculated with an ultrafiltrate prepared from pooled MChA-induced tumor tissue have shown tumor response up to the present time, that is, six months after inoculation.

A sixth GF experiment has been initiated. It is planned again to inoculate some of the chicks of this group (Dembro stock) with methylcholanthrene, but the effect of "skin painting" of the GF chicks with MChA-benzene solution will also be investigated.

The objectives outlined in the opening paragraph of this section are regarded as crucially significant to our general program of investigation in the virus field (presented and discussed in previous ONR reports). It is likely that much more attention will be given to these questions. Detailed report will be submitted after further work, possibly of a more definitive nature, has been done.

VII. COLLABORATIVE PROJECTS*

Project No. 1 - Dental Caries (with Dr. J. Blayney and Dr. F. Orland of Zoller Memorial Clinic, University of Chicago). Reported by M. Wagner in Section IV and T. D. Luckey in Section III.

Project No. 2 - Studies on the Comparative Effects of Total Body X-Radiation (with the LOBUND-AEC-ONR Advisory Committee). Reported by H. A. Gordon, W. Scruggs, M. Wagner and J. F. Reback in a special report which was presented at the Advisory Committee Meeting held at Notre Dame 22 January 1953. The report follows Section VIII.

Project No. 3 - Investigations on the Influence of the Intestinal Flora on the Infectivity and Pathogenicity of E. histolytica. (With Dr. W. Wright, Dr.C. Rees and B. Phillips of the Microbiological Institute at NIH).

The following report has been submitted by Mr. Bruce Phillips who is presently stationed at LOBUND Institute working in our Physiology Laboratory.

In 1951 a project was initiated in collaboration with the Laboratory of Tropical Diseases of the National Microbiological Institute in an attempt to further elucidate the factors of pathogenesis of Endamoeba histolytica and to obtain data on the contribution, if any, of the intestinal flora to the pathogenicity of this "so-called" pathogenic amoeba. The project was discontinued, temporarily, after one month because of complications inherent in the cultures of amebae which served as the source of inocula for experimental germ-free hosts (guinea pigs). It was found that bacteria-free cultures of the amebae were of very low virulence even for conventional (control) animals and were without virulence for such animals when they were maintained on a diet which was at that time essential to the maintenance of life in the germ-free guinea pigs.

After considerable more investigation of cultural procedures, methods were devised that permit the maintenance of bacteria-free cultures of E.histolytica of very high virulence. Furthermore, such virulence was demonstrated in conventional hosts maintained on a sterile diet which has proven more efficient in sustaining life in germ-free guinea pigs. Hence, these collaborative investigations were resumed at LOBUND August, 1952.

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* In this new section of the LOBUND-ONR Report it is planned that those collaborators who are stationed at LOBUND Institute or who have a representative here in residence will contribute a semi-annual progress report. Otherwise, progress in collaborative program will be reported by LOBUND staff members directly associated with the particular project. In the case of Dr. E. Hawk of NIH, who is stationed here, the work on ascorbic acid did not begin until late in the fall of 1952.

Up to the present time, very little data has been obtained relative to the primary objective of the amebiasis project per se due to almost innumerable complicating factors inherent in the rearing of germ-free guinea pigs. Nevertheless, most of these complications have now been obviated and thus the investigations have been of considerable value from the standpoint of information obtained relative to the maintenance of germ-free guinea pigs. The elimination of the aforementioned complications seems to ensure the ultimate success of the investigations, and there is good reason to be optimistic that the studies will contribute valuable information about the phenomenon of ameba pathogenesis.

Project No. 4 - The Study of Hemorrhagic Liver Necrosis Using Germ-Free Animals (With Dr. Paul Gyorgy and Dr. Martin Forbes of the University of Pennsylvania).

The following report has been submitted by Dr. Martin Forbes who is presently stationed at LOBUND Institute working in our Biochemistry Laboratory.

Preliminary experiments with conventional animals had indicated that the rats from the LOBUND colony were susceptible to dietary liver necrosis and that autoclaving the Himsworth diet did not in the least detract from its necrogenic character.

In the first experiment, rats with an initial weight of 85 gm. were started on the necrogenic diet. The germ-free rats survived for some 74 and 79 days on diet and their livers were found to be normal. The controls, however, did not come down with necrosis until much later, rendering the experiment inconclusive. The cause of death of at least one of the germ-free animals was found to be associated with disturbances of the blood clotting mechanism as evidenced by internal hemorrhages and delayed clotting and prothrombin time.

In the next experiment younger rats were used. Eight conventional control animals on the same autoclaved necrogenic diet as the germ-free promptly came down with necrosis in 32 days. The germ-free animals survived for 34 and 43 days. One animal had an elongated tooth and did not seem to eat well - it was found moribund on the 34th day - the animal was killed, necropsy revealed hemorrhagic areas in the lungs. The other animal was found to be moribund on the 43rd day. The animal was bled from the abdominal aorta and clotting time and prothrombin time were tested. Again a disturbance in the clotting mechanism was found to have occurred. Clotting time and prothrombin time were both delayed compared to that of conventional rats. No other pathology was observed in the rats. The livers of both rats were found to be normal.

Disturbances in the clotting mechanism were suggestive of a deficiency of vitamins, notably K. The diet, however, contained 200 mcg. vitamin K per 100 gm. and in our experience a vitamin K deficiency is not easily induced in germfree rats. Vitamin K was not greatly destroyed by autoclaving as a dietary constituent.

Since vitamin E was the only known vitamin lacking, the next experiment included two germ-free rats supplemented with vitamin E and two without supplement. Eight conventional controls again promptly came down with necrosis in about 40 days. The germ-free rats grew somewhat better

than the conventional animals. This experiment was discontinued at 69 days when a rat escaped from its cage inside the tank and chewed through the rubber glove. No abnormal pathology was observed and the livers were all normal. The growth curves show that vitamin E supplement did not alter the weight gain of germ-free animals.

The next experiment (in progress) is a repetition of the previous one. Eight out of 12 controls have died with liver necrosis in a period of 35-77 days. The germ-free animals lived 91 and 145 days and had no liver necrosis. One animal died at 91 days after a blood sample was taken from the tail and on necropsy was found to have a stomach hemorrhage. The other rat showed no pathology when the experiment was terminated at 141 days.

In the hemolysis test developed by Gyorgy and Rose, blood cells from rats deficient in vitamin E were found to hemolyze readily in the presence of dialuric acid. The germ-free non-supplemented rats were found to have negative hemolysis even after 145 days on the diet. This may indicate a high vitamin E reserve; however, the alterations in blood clotting mechanism were at least partially remedied by supplementation of vitamin E. Since supplements of vitamin E to the animals cause an observable change it is probable that non-supplemented animals did not have full stores of vitamin E.

These experiments show that germ-free rats do not die with hemorrhagic liver necrosis within the time during which all conventional control rats died with liver necrosis when fed the same autoclaved necrogenic diet.

Project No. 5 - The Role of Ascorbic Acid (or Antibiotics) Replacing the Rat's Requirement for Pantothenic Acid. (With Dr. F. Daft and Dr. E. Hawk of the Institute for Arthritic and Metabolic Diseases at NIH)

Refer to Section III - Biochemistry and Nutrition.

VIII. SUMMARY

I. Administrative Section

The status personnel employed on the Task Order, alterations of physical plant, publications, contract status, and equipment needs are discussed.

II. Germ-Free Life Production Section

Changes in apparatus and techniques are listed. A report is given on the production of germ-free animals during 1952.

III. Biochemistry and Nutrition Section

The biochemical survey has indicated very few major differences between germ-free and conventional chickens. Although the survey of the rat is "in progress" the general pattern appears to be similar.

Several diets have been used in attempts to obtain a good sterilized diet for maintaining a stock colony of germ-free rats. The synthetic diets work well before and after sterilization. There is still room for improvement in the practical diets. Germ-free chicks appear to have the same qualitative vitamin requirement as the conventional chick. The work on the effect of feeding antibiotics is still "in progress".

The work of the diet kitchen appears to be rapidly increasing both in types of diets and quantity of diet produced.

The work in five collaborative projects has progressed satisfactorily.

IV. Bacteriology and Serology Section

The Bacteriology Laboratory ran sterility testing on all animals raised in germ-free type apparatus as a service to the various projects at LOBUND.

Preliminary work to determine the approximate time at which post-mortem invasion takes place into the peritoneal cavity of the conventional non-X-rayed albino rat showed that generally, the peritoneal cavity will test sterile up to 10 hours after death. The incidence of positive peritoneal cavity cultures increases after 10 hours post mortem but a negative test was still observed at 18.75 hours after death.

A pilot experiment on the ability of bacteria and bacterial products to penetrate the wall of an isolated intestinal loop in a germ-free and conventional rat was run. No data are submitted at this writing.

In the dentalcaries project, four germ-free rats failed to produce carious molar lesions after 150 days on a diet which in conventional animals produces a high caries incidence. Four rats harboring only one single strain of bacteria, namely Lactobacillus #465, also failed to develop gross carious molar lesions. Their counterparts, four rats brought into the conventional animals room environment, all developed caries after 150 days on the same diet. Bacterial counts made from oral swabbing taken from the mouth of the latter two animal groups showed the average total count of bacteria per gram oral sample to be >1000 times greater in the conventional rat as compared to the average count taken from the mono-contaminated group. Similar studies are now underway using Streptococcus liquefaciens as a possible etiological agent in dental caries.

Quantiative counts were made on the contents of higher and lower gastro-intestinal segments of rats harboring only single strains of bacteria. Lactobacillus monoflora rats showed a considerable increase in numbers of organisms per gram contents in the lower segments. This increase in numbers from higher to lower segments was not nearly so marked in the Bacillus cereus monoflora group. The count in the cecum and colon of the Bacillus cereus group was considerably lower than counts from the same intestinal segments in the Lactobacillus monoflora group and in the conventional (polyflora) group.

The Universal Serologic Reaction applied to germ-free and conventional chicks showed:

1. Zero to 1 day old chicks do not react.
2. Older chicks do react.
3. Germ-free chicks tend to react less in the 0 and 0.15% NaCl titrations than do conventional chicks.
4. Germ-free chicks generally tend to react somewhat less over the entire NaCl concentration range from 0 to 2.1% than do conventional chicks.
5. Point 3 above represents only a trend, since aberrant results negate a distinct qualitative difference between the germ-free and conventional groups.

Experiments to determine antibody response to parenterally injected antigens in germ-free and conventional chickens are currently underway.

V. Physiology and Pathology Section

1. Intestinal absorption and secretion, as well as intestinal bacterial penetration were studied in germ-free, conventional and control-contaminated rats.

2. Leukocyte concentrations were determined in the venous blood of various organs together with arterial leukocytic level in the same animal material.

3. Intestinal fat absorption was studied in cesarean-born baby rats during the first hours of life.

4. Collaborative projects (NIH amebiasis and AEC) are summarized in special reports.

5. Observations were made in autopsied germ-free animals lost from the LOBUND colony.

6. Efforts were made to initiate a disease-free conventional rat colony.

VI. Virology Section

Summaries have been presented covering nine projects currently under investigation, including studies on lymphomatosis virus, Theiler's encephalomyelitis virus, human infectious hepatitis virus, virus-microbe associations, latent viruses, symbiotic viruses, a neurogenic disease of GF chicks, a long condition in GF rats, and filtrate passage of methylcholanthrene-induced tumors.

VII. Collaborative Projects Section

The five major collaborative projects now being investigated under this Task Order are listed and progress reports are included or referred to in other sections.

ADDENDUM

A special report "Study on Comparative Effects of Total Body Radiation in LOBUND Albino Rats - Germ-Free and Conventional II". is included.

STUDY ON COMPARATIVE EFFECTS OF TOTAL BODY RADIATION IN LOBUND ALBINO RATS - GERM-FREE AND CONVENTIONAL - II.*

I. Introduction

II. Methods and Materials

1. Animal Material
2. Bacteriological Methods of Testing for Sterility
3. Autopsy, Hematological and Histological Techniques
4. Irradiation Data

III. Results: Germ-Free and Conventional Rats Exposed from 300 r to 1000 r.

1. Clinical Observations
2. Hematological Observations
3. Autopsy Protocols
   A. Summaries of previously reported protocols
   B. Recent protocols
4. Histological Reports
   A. Previously reported summaries
   B. Recent reports

IV. Addenda

1. A Study on the Effects of Total Body Radiation on Contaminated, Previously Germ-Free Rats.
   A. Streptococcus group - 800 r
   B. Casecolyticus-like group - 800 r
   C. B. cereus group - 800 r
   D. Micrococcus ureae group - 2000 r

2. Exploratory Experiments
   A. The effect of post-irradiation streptomycin treatment on germ-free rats
   B. Concerning latent and symbiotic viruses in germ-free animals
   C. Post-mortem bacterial invasion into the peritoneal cavity of the conventional Albino rat

3. Minutes of the LOBUND - AEC Advisory Board Meeting, December 17, 1951.

V. General Summary

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* In this Report the word "conventional" designates the normal contaminated animal, while "contaminated" characterizes the previously germ-free animal which lives in conditions of controlled contamination.

I. INTRODUCTION

In the original proposal to AEC, 20 April 1950 from LOBUND Institute, the objective was set forth as, "The use of germ-free and control animals in the study of radiation sickness". An amendment (Phase 2) to Contract N6-ori-83, Task Order 3, became effective 1 July 1950. It is under this broad objective that we have worked.

From the start of the work, after consultation with the LOBUND staff, it was decided to invite a small group of experts in the field of radiation sickness as consultants. Accordingly, invitations were issued to the following: Dr. George LeRoy, University of Chicago; Dr. C. Phillip Miller, University of Chicago; Commander Eugene Cronkite, NNR Institute; Dr. Jon Howland, University of Rochester; Dr. Charles Dunham, AEC and Dr. Roger Reid, ONR. These invitations were graciously accepted and this group has not only advised but participated in the work.

Within the framework of the original directive, certain steps or phases became evident which required a narrowing pro tem of the original objective without, however, cancelling it. Thus, on 24 October 1951 the immediate objective was divided into three phases: (A) Studies on the nature of radiation disease in germ-free animals; (B) Studies on the nature of radiation sickness in germ-free animals which have been selectively contaminated and (C) Studies on inhibitors in phases A and B above. On 6 September 1951, in an amendment, the immediate objective was stated to be an extension of Phase 1 to include "The study of the evaluation of the role of bacterial infection in the outcome and pathogenesis of the syndrome of radiation injuries". The proposal of 9 May 1952 had as a stated objective "to determine whether the germ-free condition has any effect on the survival time of total body X-irradiated rats and to determine whether controlled contamination (mono- or di-contaminated animals) has any effect on the survival time of total X-irradiated rats". It must be noted that it took one year for building alteration, setting up the X-ray and calibrating it. Thus, the work reported herein has been underway less than two years.

Progress involving the use of germ-free animals and techniques is necessarily slow because of certain limitations due to: need for breeding and rearing animals to a certain weight (Ca. 250 grams) which roughly covered a period of 18-22 weeks of which 10-14 weeks covers the period from birth to useful weight; the length of time from set-up to termination (approx. 75 days) of an experiment; limited number of germ-free units in which the animals could be housed during experiment; limited number of germ-free cages set aside for breeding the colony; accidental contamination (an always and ever present factor where the invasion of a single microbe spells termination); and finally the small number of animals which can be housed in a single germ-free unit. Nevertheless, in spite of this we have managed to provide 38 germ-free animals and 19 animals reared germ-free and mono- or di-contaminated. Also to be considered is an accident due to failure of a building temperature control, which in November 1951 cost us the entire germ-free colony due to an excessive temperature of 125°F.

This report covers the period between December 1951 to December 1952 and for purposes of discussion includes an overall summary of the work completed.

are

The experimentally contaminated rats normally referred to as contaminated (by intention or accident) at a designated age with one (mono-contaminated), two (di-contaminated) or more (poly-contaminated) strains of microorganisms. It must be further noted that once a germ-free rat becomes contaminated and is used experimentally the specified strains of microorganisms and no others make up the rat-microorganism complex during the experiment reported.

This study has had to be necessarily exploratory since this is the first time germ-free animals have been used in the study of radiation sickness. Thus, it has been necessary to establish a base line from which to work comparatively. We feel that the base line has with this report been indicated and is now ready for discussion.

It might be well to point out that comparisons between germ-free and conventionally contaminated animals are only in the broad sense one of difference between the presence or absence of microbes. It should be remembered that a germ-free animal has never had experience with living microbes, whereas the conventional animal spreads this experience over a period between birth and the termination of an experiment. Thus, the conventional animal at the time it is used for experimental purposes has been conditioned by this experience and certain systems have been activated which are not fully activated in the germ-free animal of comparable age. How much difference this makes in comparative results is difficult to assess but must be considered. This difference might be alleviated to some extent if two conventional animals were used, one of which was freed from all microbial contamination at the time of the experiment, but this has not been possible to date.

We have anticipated by our studies describing the germ-free animal per se the need for a comparative base line. It is from this base line and within the framework of the germ-free state as it exists that the problem of radiation sickness has its greatest implications. The study of radiation sickness in a comparatively pure biological system, uncomplicated by vicarious and indiscriminate microbial experience, should be first accomplished in any program seeking to evaluate the role of microorganisms or the exact progress of the syndrome.

In these studies, time has narrowed the directive given originally to that of comparisons between the survival time of germ-free and conventionally contaminated controls from the same strain of animals, maintained on the same diets and irradiated in an equivalent manner. A difference in survival within the range of dosage selected is indicated. Whether this difference is due to the total absence of and experience with microbes on one hand, or the flora as it exists in these conventional controls, cannot be said with certainty. For example, what would be the situation in a challenge experiment with low grade pathogens in the conventional animal or what would alter the survival time in germ-free animals?

This report includes a number of exploratory studies which were set up in keeping with later directives. This approach should be expanded and the attention of the committee is directed to this point. Indicative here are studies involving animals other than rats. The rat is at best a less desirable animal with respect to bacterial invasion and antibody formation. For instance, the failure to detect bacteremia, show post-mortem invasion, lack of protection by antibiotics (aureomycin, polymyxin, terramycin, penicillin), etc. The nature of the rat species may well account for the differences observed between the rat and mouse. On the other hand, the syndrome

in the rat is not markedly different than that in other animals, and if microbial invasion plays a less important role in the rat, the rat still is a valuable animal in which to study the systemic syndrome.

II. METHODS AND MATERIALS

1. Animal Material

Animal material consisted of 22 germ-free and 21 conventional rats. Two of the germ-free animals were 1st generation germ-free (i.e., Caesarian-born, hand-fed through weaning) while the others were 3rd, 4th, and 5th generation germ-free. All animals were reared from weaning on a steam-sterilized, semi-synthetic diet. Table I shows the composition of the diet.

TABLE I.
Composition of Diet I.-128

CONSTITUENTS	AMOUNTS
casein (Labco)	20 g
rice (polished)*	63 g
Salts II (F free)	5 g
"Crisco" **	5 g
cellophane spangles (DuPont)	3 g
Vitamin A	800 IU
Vitamin C	200 mg
Vitamin D_3	100 IU
Vitamin E	50 mg
Vitamin K	10 mg
thiamin Cl	6 mg
riboflavin	3 mg
pyridoxin HCl	2 mg
Ca-pantothenate	30 mg
nicotinamide	5 mg
choline Cl	200 mg
inositol	100 mg
p-aminobenzoic acid	5 mg
biotin	0.1 mg
folic acid	1.0 mg
nicotinic acid	6 mg
B-pyracin Cl	1 mg
pyridoxamine Cl	0.4 mg
yeast extract (Difco)	2 g
whole liver powder (Armour)	2 g
corn oil (Mazola)	1.6 g
corn starch	0.5 g

* particle size between 15 and 60 mesh

** This hydrogenated vegetable oil is a product of the Procter and Gamble Co. of Cincinnati, Ohio.

2. Bacteriological Methods of Testing for Sterility.

Sterility tests were run on a variety of samples taken from the animal itself and from various substances and surfaces within the cage. Each sample was taken on a sterile cotton swab and submitted to the bacteriological routine either inside the cage itself or brought out to the laboratory for more detailed observation.

The samples were taken from the following sources:

(1) feces and urine
(2) liquid and solid food
(3) drinking water and water used to wash and clean equipment
(4) swabbings from mouth, anus, and other orifices
(5) swabbings from fur and skin surfaces
(6) swabbings from interior cage surfaces such as walls, air inlet and outlet, gaskets and surfaces of the rubber gloves and cage equipment

For direct microscopic examination, wet mounts were prepared and observed for the presence of bacterial, fungal and protozoan forms. Smear preparations were also made, stained by the Gram technique and observed microscopically.

Samples were cultured on two main types of media: (1) brain-heart infusion agar enriched with 5% sterile defibrinated normal horse blood and (2) fluid thioglycollate medium.* Multiple swabbings were taken from each sample source previously listed, so that replicate numbers of plates of solid media and tubes of liquid media could be inoculated with each sample source to be tested.

Petri plates were prepared aerobically and anaerobically and incubated at various temperatures; room temperature (approximately 25° C.), 37° C and 55° C.

Fluid thioglycollate tubes provided a gradation of oxidation-reduction potential and were incubated at the same three above mentioned temperatures.

All cultures were observed periodically up to two weeks for appearance of growth. Blood agar plates were examined under 90 X magnification and Gram stained preparations were made from thioglycollate tubes before discarding.

The data presented for the germ-free rats represents results obtained from animals showing no evidence of viable microbial associates according to the test methods employed.

The sterility testing procedures do not answer the difficult question of whether the animals were also virus-free. However, since virus detection depends mainly on an outward manifestation of disease or pathological process, there was no reason to believe that the germ-free groups harbored any active virus.

* The solid medium was Bacto-Brain-Heart Infusion with 1.5% agar added, from the Difco Laboratories, Inc., Detroit, Michigan, whereas the fluid thioglycollate medium was from the Baltimore Biological Laboratory, Inc., Baltimore, Maryland.

3. Autopsy, Hematological and Histological Techniques

These were unmodified from those reported to the LOBUND Advisory Board meeting on December 17, 1951.

4. Irradiation Data

The animals were all exposed to total body x-irradiation with a Picker 260 KVP therapeutic unit. The physical factors were: T.S.D., 50 cm; 15 ma; 250 KVP at 15 r/minute measured in air and administered in a single exposure. A filter of 0.25 mm. Sn, 0.4 mm Cu, and 1 mm Al was used at the master cone, and after leaving this filter the beam was projected through an aluminum port 1 mm thick. The rats were individually restrained in annular cylinders of aluminum which were about 1 mm thick. The same cylinders were used for both conventional and germ free rats.

III. RESULTS

1. Clinical Observations

Germ-free and conventional rats at all radiation levels continued to eat and take water immediately after and for at least 48 hours following exposure. These rats appeared to become anorectic only terminally and this was observed to occur concomitantly with the onset of diarrhea in all conventional rats while positive evidence of diarrhea was noted in but one germ-free rat exposed to more than 400 roentgens.

In general it may be said that the conventional rats were in a moribund state over longer periods of time than were the germ-free rats; this difference was particularly noticeable in the animals exposed to 1000 r. The germ-free rats would weaken and die within a period of 5 hours while the conventional rats lay in a profound shock-like state for as long as 24 hours before dying.

Weight changes for both groups are shown in the accompanying curves (Fig. 1).

Germ-free rats which were exposed to more than 400 roentgens showed many ecchymotic areas visible from the surface of the skin; this type of lesion was not observed in conventional animals as a rule presumably because the survival time of these animals was considerably shorter than that of the germ-free rats. In addition, conventional rats exposed to 400 roentgens and more usually showed bloody, encrusted deposits about their eyes and nostrils from about 24 hours post-irradiation until they died; the germ-free counterparts of these rats usually did not show these encrustations. One 1000 r germ-free animal and one 700 r germ-free animal were noted to have encrusted nostrils terminally, however, this material seemed more serous than hemorrhagic in origin.

Intra-ocular opacities occurred in both groups of animals to the same extent and these could not be correlated to the radiation dose.

While the number of germ-free animals thus far irradiated is insufficient to fix precisely the LD 50/30 days, our data indicate it to lie between 600 r and 700 r for germ-free animals. Similarly the data indicate that the LD 50/30 days lies between 300 r and 400 r for conventional animals reared under suitable control conditions. In lieu of a survival-dose curve we submit detailed survival data (Fig. 2 and 3, Table II) which compares the survival time of total body x-irradiated germ-free rats with the survival time of similarly exposed conventional animals. These data demonstrate that at severe radiation levels (in the range 400 r to 1000 r) germ-free rats survive twice as long as similarly irradiated conventional animals.

Weight Curves of Individual Irradiated Rats

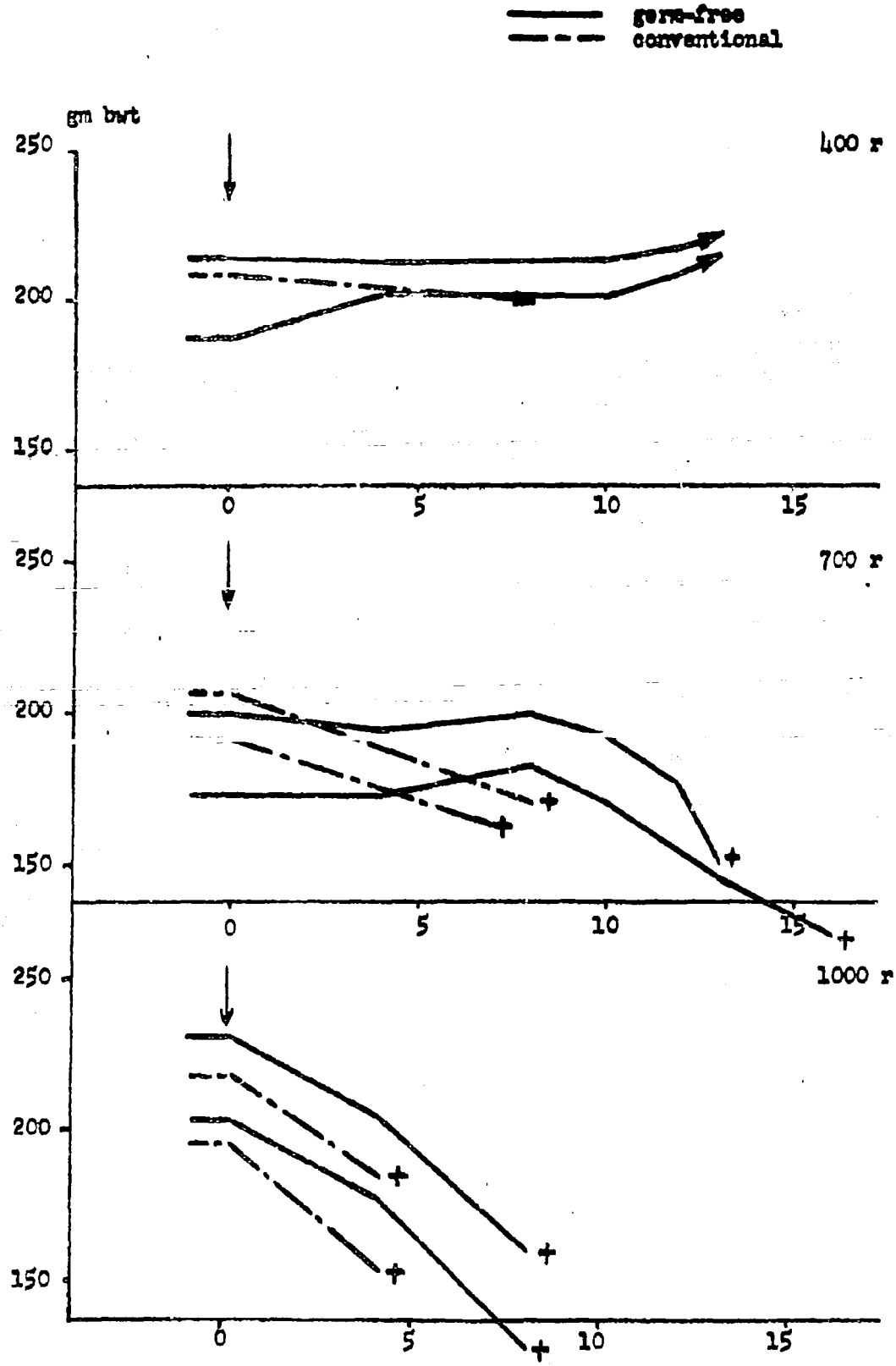
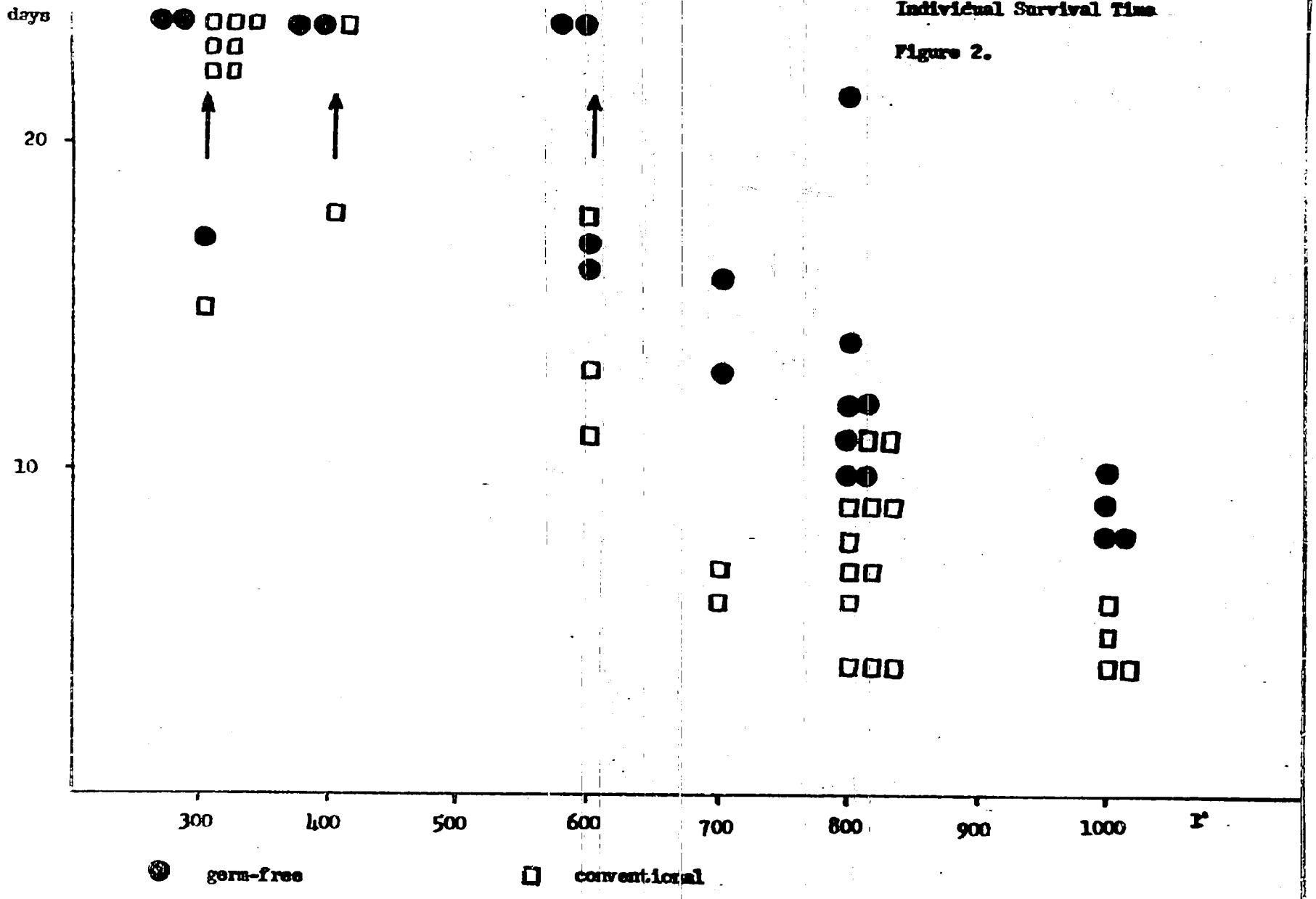


Figure 1.



Individual Survival Time

Figure 2.

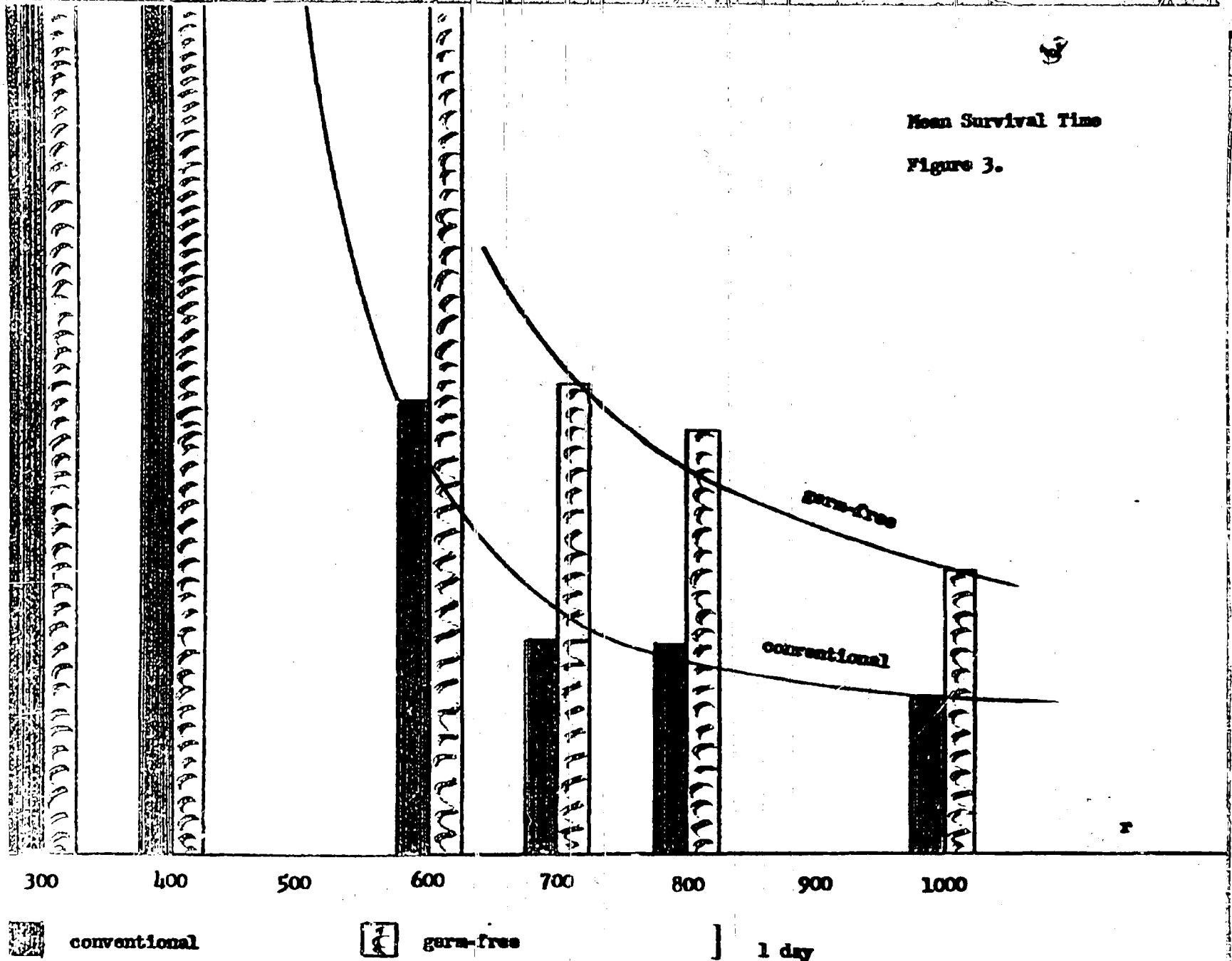

Mean Survival Time
Figure 3.
germ-free
conventional
300
400
500
600
700
800
900
1000
conventional
germ-free
1 day

TABLE II

GERM-FREE				CONVENTIONAL CONTROLS			
No.	Date Exposed	Dose (Roentgens)	Survival Days	No.	Date Exposed	Dose (Roentgens)	Survival Days
1001	12/5/52	1000	8	1003	12/23/52	1000	4
1002	12/5/52	1000	8	1004	12/23/52	1000	5
1033	4/16/52	1000	10	1035	4/16/52	1000	6
1034	4/16/52	1000	9	1036	4/16/52	1000	4
814*	8/27/51	800	22	800(6)*	9/17/51	800	8.8
817	1/10/52	800	10	819	1/10/52	800	7
818	1/10/52	800	12	820	1/10/52	800	6
833	4/16/52	800	14	837	4/16/52	800	4
834	4/16/52	800	12	838	4/16/52	800	8
835	4/16/52	800	10	839	4/16/52	800	7
836	4/16/52	800	11	840	4/16/52	800	4
701	12/5/52	700	13	703	12/23/52	700	7
702	12/5/52	700	16	704	12/23/52	700	6
617	1/10/52	600	>62	619	1/10/52	600	18
618	1/10/52	600	17	635	4/16/52	600	11
633	4/16/52	600	>62	636	4/16/52	600	13
~~634~~	~~4/16/52~~	~~600~~	~~16~~				
401	12/5/52	400	>40	403	12/23/52	400	>28
402	12/5/52	400	>40	404	12/23/52	400	18
313*	8/27/51	300	>30	300(6)*	9/17/51	300	>80
~~333~~	~~4/16/52~~	~~300~~	~~17~~	~~335~~	~~4/16/52~~	~~300~~	~~15~~
334	4/16/52	300	>62	336	4/16/52	300	>62

Average Survival Times

1000 r --- 8.8 Days
800 r --- 13 Days
700 r --- 14.5 Days
600 r --- >30 Days
400 r --- >30 Days
300 r --- >30 Days

Average Survival Times

1000 r --- 4.8 Days
800 r --- 6.4 Days
700 r --- 6.5 Days
600 r --- 14 Days
400 r --- >30 Days
300 r --- >30 Days

* Rats #313 and #814 were 1st generation germ-free while others here Reported were 3rd, 4th, and 5th generation germ-free.

There were 6 conventional controls in each of these groups. At the level of 800 r the individual surviving times were: 11, 11, 9, 9, 9, and 4 days. All 300 r conventional rats irradiated at the same time survived for at least 30 days.

2. Hematological Observations

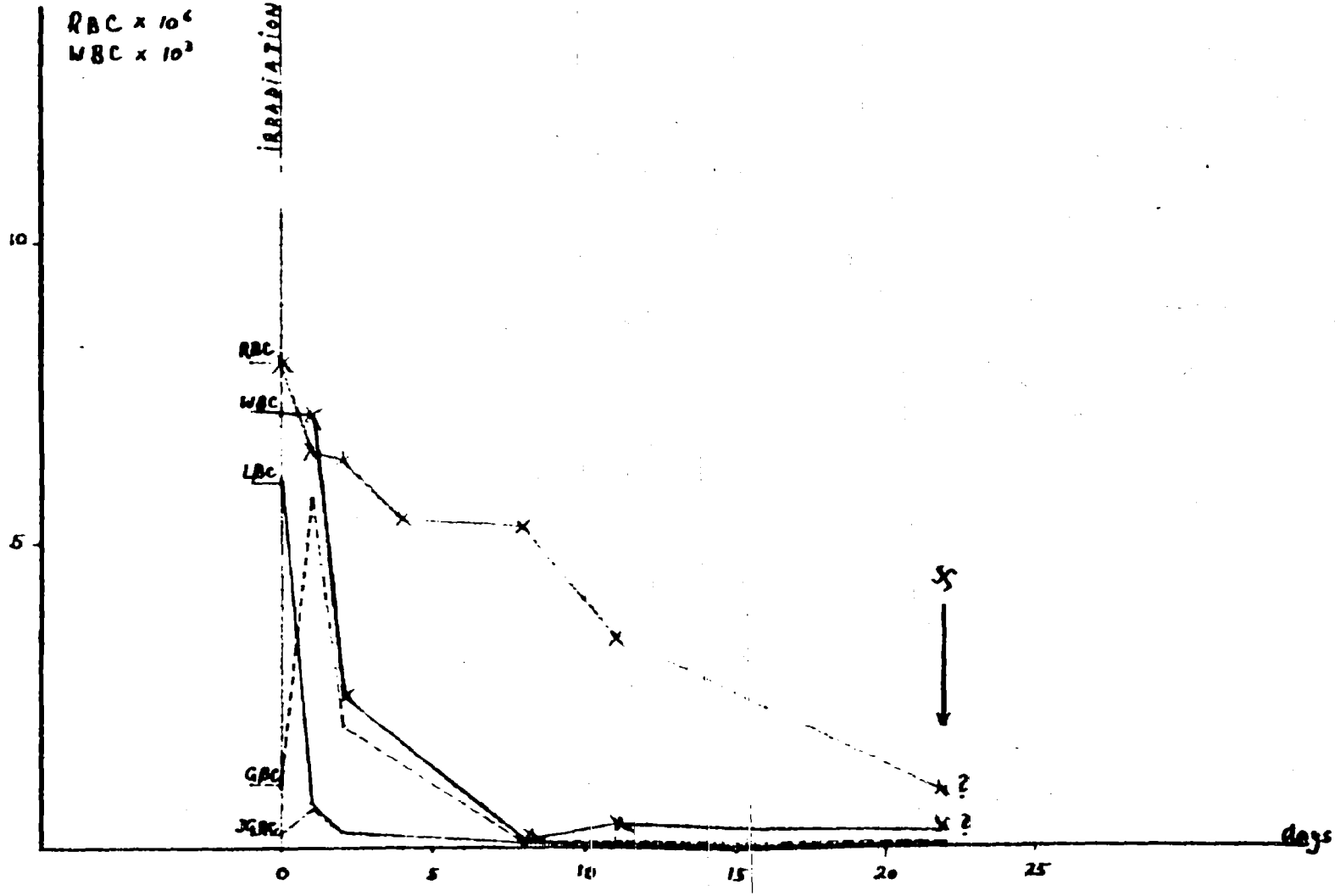
According to the LOBUND-AEC Advisory Board recommendations, no hematology was taken in the recent runs. In order to summarize previous results, four RBC and WBC curves are given in this report presenting four rats, germ-free and conventionals, irradiated at 300 r and 800 r (figs. 4, 5, 6, 7).

Legend:

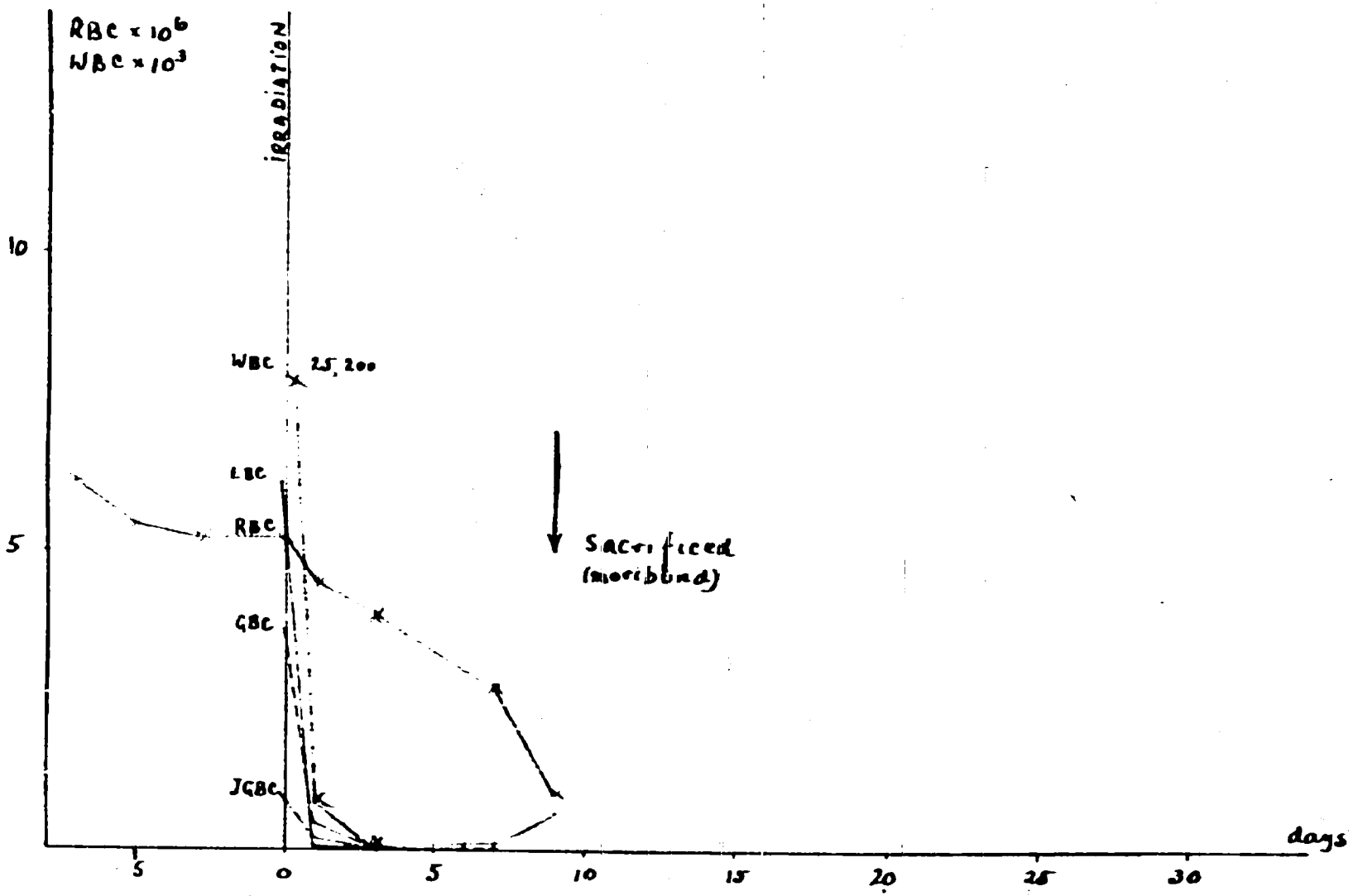
RBC = Red blood count
WBC = White blood count
GBC = Mature granulocytes
LBC = Lymphocytes
JGBC = Immature granulocytes

(All values are presented in absolute figures.)

53LoB ♀-3 # 814 ♂ germ-free 800 r

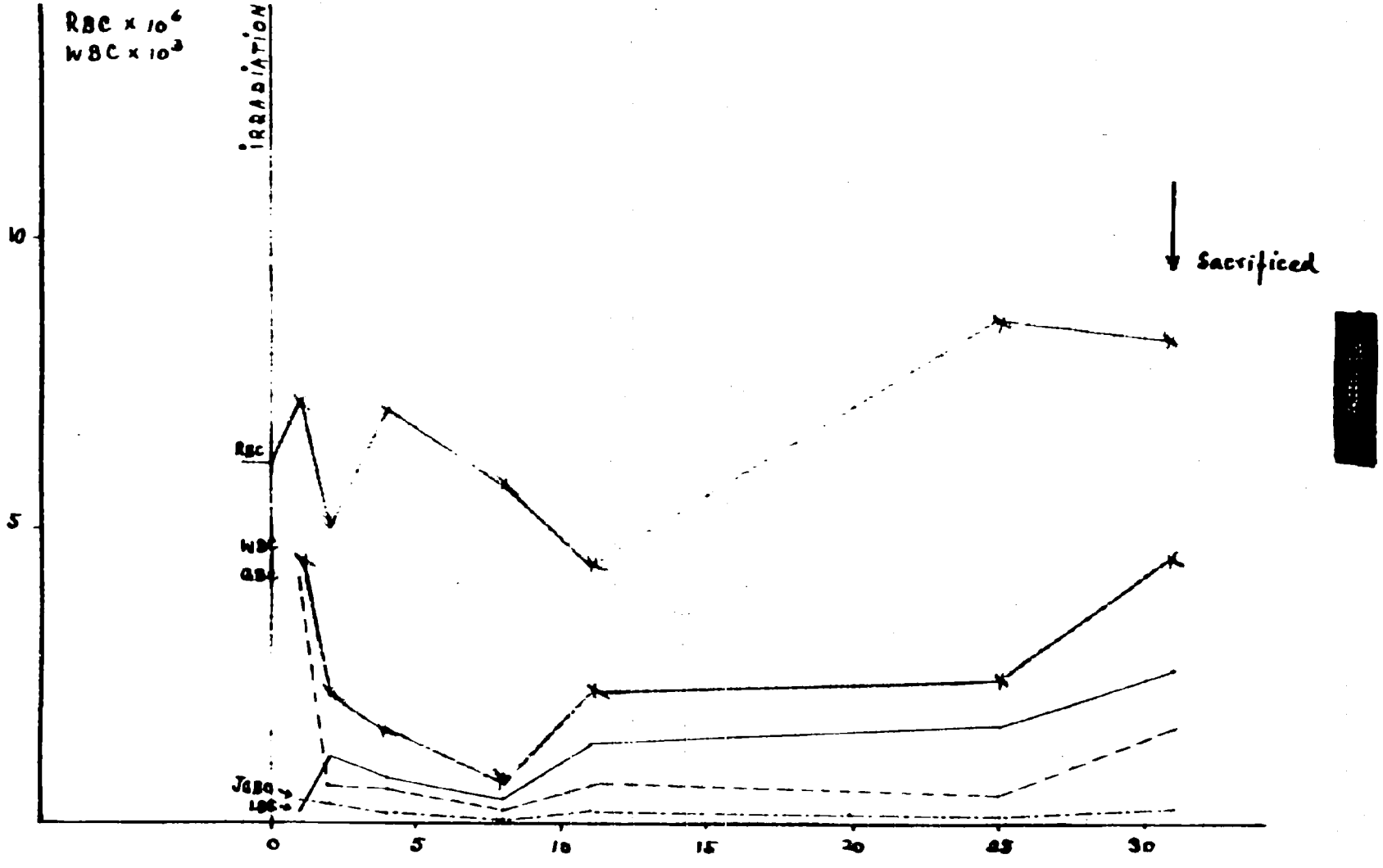


53 LOBL-3 # 807 ♀ [redacted] 800 r

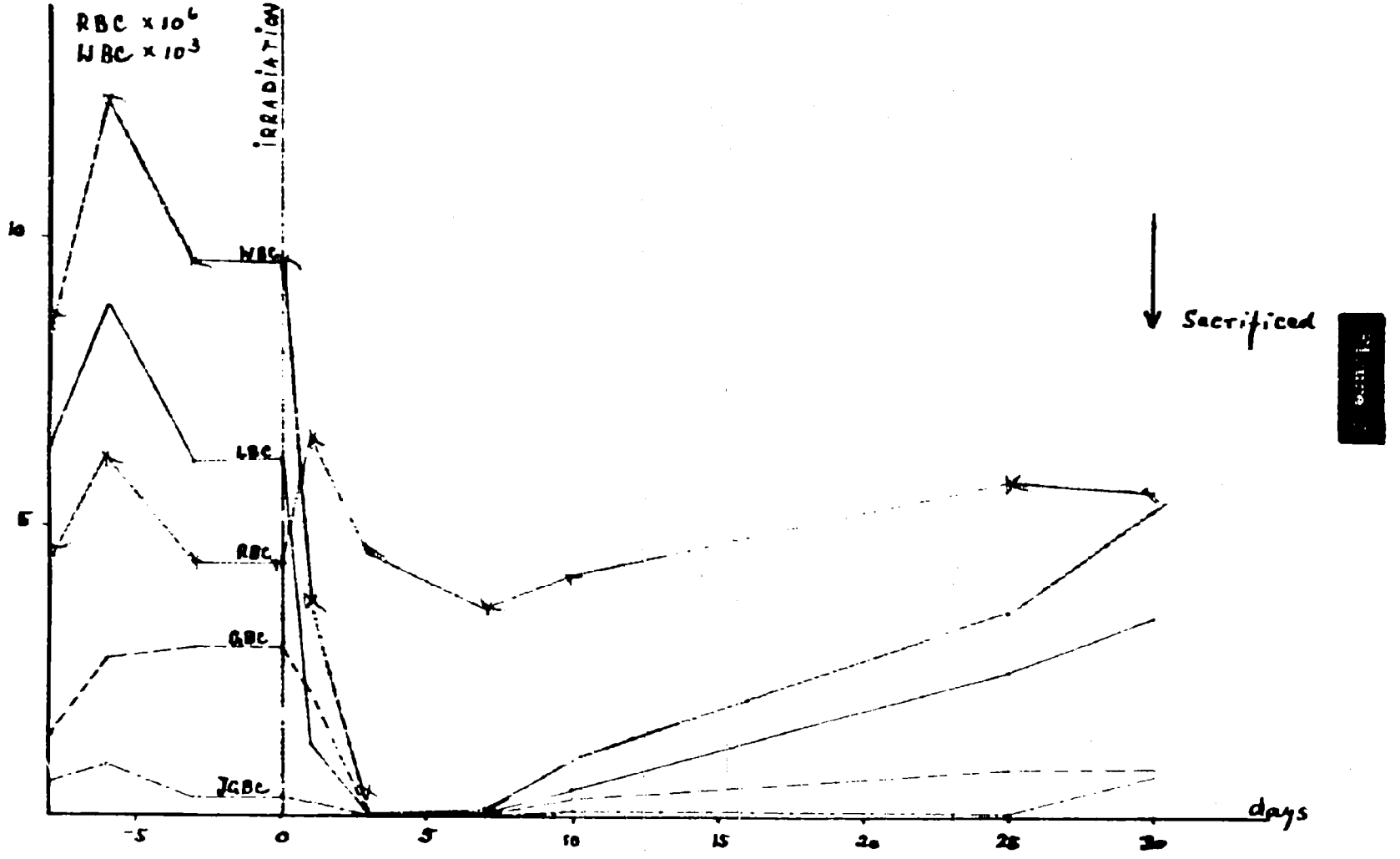


[redacted]

53 LOB 2-3 # 313 ♀ germ-free 300 r



53L082-3 # 301 ♀ [redacted] 300r



3. Autopsy Protocols

A. Summaries of Previously Reported Protocols

With the exception of rat #814 germ-free which died abruptly the 22nd post-irradiation (800 r) day with an overwhelming pulmonary edema (a white, frothy, free flowing liquid completely filling the trachea), the 800 r animals, both germ-free and conventional uniformly showed occasional intra-ocular opacities and a tendency towards epilation, with many ecchymotic areas in the subcutaneous tissues; markedly atrophic thymi and hyperemic lungs with sero-sanguinous exudates in the pleural cavities; yellowish brown livers and severely hemorrhagic pancreases (not obvious in #814); many petechiae and erosions in the glandular portions of the stomachs and occasional pin-point hemorrhages along the serosal surface of the intestine; and hemorrhagic lymph-nodes were observed in both thoracic and peritoneal cavities. In conclusion, the overall picture seen in both groups was the same in kind, differing in degree in that the conditions seen in the conventional animals were considerably intensified in the germ-free animal.

The 300 r rats were sacrificed after 30 days, and except for a tendency towards hyperemic lungs, atrophied thymi and occasional cataracts (rat #313, germ-free, had cataracts in both eyes), these animals showed no macroscopically visible abnormalities.

B. Recent Protocols

Four additional autopsy protocols of recently irradiated rats are submitted:

63H10-2A-#1002 ♂
Germ-Free. Irradiated 8 December 1952 (1000 r)
Died 13 December 1952-Weight 162 gms.

There were intra-ocular opacities in both eyes and the animal's hair was rather easily pulled out. There was evidence of an exudate about the animal's nostrils and eyes.

The subcutaneous tissues show many ecchymotic areas.

The chest contained about 0.5 ml of a sero-sanguinous exudate. The lungs were extremely hyperemic with occasional very dark reddish-brown areas suggesting recent infarcts. The pericardium contained what was considered a slightly excessive volume of pericardial fluid which was pale reddish-brown in color. The surface of the myocardium seemed roughened though it glistened.

The liver was of a strikingly pallid brownish-yellow color. The stomach showed many petechiae on the serosal surface while the mucosal surface at the margin of the glandular portion of the stomach showed several blister-like bullae which on sectioning appeared to be filled with blood clots. The intestine appeared normal grossly but did not contain any chyme, nor did the colon contain fecal material. The pancreas, mesentery, and mesocolon were profoundly hemorrhagic. The spleen was flabby and brownish-red in color.

The lymph-nodes of the chest and abdomen were hemorrhagic.

Both adrenal glands seemed edematous while the right adrenal showed several cortical pin-point hemorrhages.

The testes were flabby and extremely hemorrhagic.

The brain was normal.

53H10-2B-#1003♂
Conventional Irradiated 23 December 1952
Died 27 December 1952 - Weight 155 grams

The animal's hair was not particularly easy to pull out. There were many reddish-purple spots visible through the skin, and these when the skin was opened were seen to be rather large (about 1 sq. cm.) hemorrhagic areas.

There was an intra-ocular opacity in one eye while the other appeared normally colored but had several fine, bloody streaks which seemed to be floating in the eye. There was a red crusty deposit about both eyes and the nose.

The thymus gland was somewhat atrophied. The lungs were severely hyperemic throughout the parenchyma with noticeable areas of hepatization around the free margins.

The liver showed many very yellowish-brown spots throughout parenchyma, although the liver as a whole was not nearly so yellow as that of rat #1002. The stomach showed many petechiae and the pancreas and mesentary were markedly bloody in appearance.

The adrenal glands were essentially normal.

The kidneys showed no outstanding changes. The testes were of a bluish color and profoundly soft. The lymph-nodes wherever visible were heavily engorged with blood.

The peritoneal abdominal fat showed many areas of severe fat necrosis.

53H1-2A-702♂
Germ-Free. Irradiated 5 December 1952 (700 r)
Died 21 December 1952 - Weight 128 gms.

A corneal opacity was in the left eye, but it was felt that this might have been a post-mortem change in that the eye seemed much more dry than the right member. No ecchymotic areas were visible through the skin and the hair was very easily brushed from the animal.

There were several brownish-black and several reddish-brown areas on the surface of the lungs. These gave the impression of representing both recent and old infarcts. The heart seemed rather hemorrhagic and slightly roughened. The liver was very pale and its surface seemed much more sponge-like in texture than was seen in any other of the animals.

The stomach showed many severely ecchymotic areas. A noteworthy finding in this animal in contrast to others in this series was that there were no hemorrhages in either the mesentery or the pancreas. The spleen was very soft and very brown in color.

53H100-2B-#703 ♂
Irradiated 23 December 1952

This animal's autopsy protocol is essentially like that of 53H1-2A #702. It differs from it in that this animal's lungs were much more hyperemic and the pancreas appeared to be a single wide spread blood clot. In addition, there were reddish-brown encrustations about the eyes and nose and evidence that the animal suffered from a severe diarrhea.

4. Histological Reports

A. Previously Reported Summaries

(a) NIH 55789 — LOBUND 55LOB2-3 #313
Germ-free 300 r

The hemorrhages in lung, adrenal, lymph nodes, and the large amount of hemosiderin in the spleen are probably due to the radiation injury. The significance of other changes cannot be evaluated in the absence of non-irradiated germ-free, control animals. (Normal amount of erythropoiesis in spleen? Cellularity of bone marrow? Normal size and structure of lymph nodes?).

The kidney lesions are not considered due to radiation injury, since similar changes (except for the globular inclusions in epithelial cells) were absent from the irradiated germ-free animal given 800 r, and because experience on non-germ-free animals indicates a relatively high degree of resistance of the kidney to radiation.

(Cronkite and Brecher)

The sections showed no lesions which could be attributed to irradiation. The lymphoid tissue of the intestinal tract and lungs was reduced in amount, however, there was active lymphopoiesses on the spleen and lymph nodes.

(Howland)

(b) NIH 55790 — LOBUND 55LOB2-3 #314
Germ-free 800r

Although the bone marrow from the femur was unfortunately not present in the sections of the femoral bone, the appearance of the spleen with extensive deposition of hemosiderin strongly suggests that bone marrow regeneration was minimal. (As a rule hemosiderin disappears from the spleen when bone marrow regeneration gets underway). Moreover, in the spleen itself erythropoiesis is rather slight and most of the regeneration is still in the stage of stem cells. It therefore appears likely that lack of bone marrow regeneration was responsible for a severe anemia, resulting in extensive fatty infiltration of heart and liver, and that the anemic anoxia was responsible for the edema of the lung. The nature of the abnormality of the periportal liver cells (increased size of nuclei and homogeneity of cytoplasm) is not clear.

(Cronkite and Brecher)

The spleen was devoid of lymphoid tissue except for a few cells about the arterioles which showed an occasional mitotic figure. The lymph node was congested and contained pigmented phagocytes; there were a few lymphocytes in the cortex but no follicles. The mucosa of the gastro-intestinal tract, except for post-mortem autolysis, appeared normal.

(Howland)

(c) NIH 55791, 55792 LOBUND 53LOR2-3 #807 & 812
Conventional - 800 r

(807) Bronchiectasis with mononuclear inflammatory reaction. Hemorrhage, erythrophagocytosis and pigment deposition in lymph nodes; no regeneration of lymph follicles. Incompletely regenerated thymus. Extensive hemosiderosis and virtually no regeneration of follicles in the spleen.

(812) Similar to 807. Only some diffused regeneration of lymphoid elements in the lymph nodes. Several petechiae in mucosa of stomach. Largely lacking spermatogonia; no mitoses in spermatocytes.

(Cronkite and Brecher)

The spleen of rat 807 contained only a few lymphocytes about the arterioles. There were a number of lymphocytes in the lymph nodes but no active nodules were seen. One node was congested and a number of pigmented phagocytes were present. The thymus was cellular and appeared to be involuting. The ovaries contained a number of active follicles. There were no lesions of the intestinal mucosa.

In general the lesions of rat 812 were much the same as in No. 55791. The seminiferous tubules of this rat showed active spermatogonia were markedly reduced in number.

(Howland)

(d) General Comment

All 3 animals exposed to 800 r (1 germ-free, 2 control) show hemorrhages into lymph nodes and elsewhere. All 3 animals exhibit marked splenic hemosiderosis and little or no evidence of regeneration of splenic hemopoiesis; such an appearance of the spleen is found only when bone marrow regeneration is minimal or absent. It may therefore be inferred, that all 3 animals had little or no regeneration of hemopoiesis.

The anemia in the germ-free animal exposed to 800 r, as judged from the extensive fatty infiltration of heart and liver was probably much more severe than in the controls. A longer survival of the germ-free animal in the absence of bone marrow regeneration would be a possible explanation.

(Cronkite and Brecher)

The histologic response of the two germ-free rats was approximately what one would expect at the dosage given; namely, recovery by the 30th day of the 300 r animal and definite radiation changes in the animal receiving 800 r.

The lesions in the controls are confusing and are not in accord with the usual findings at a dosage of 800 r. The depletion of lymphoid tissue is about all one sees to indicate that the animal received irradiation. The absence of more severe lesions of the gonads would imply that these organs had not received 800 r.

(Howland)

B. Recent Reports

(a) NIH Pathology No. 58863 — LOBUND No. 53 H 7-2A #617
Germ-free - 600 r

Spleen: The Malpighian follicles are well developed and there is a broad zone of larger lymphocytes or reticulum cells. There are scattered small foci of erythropoiesis in the pulp and there is an approximately normal amount of hemosiderin in macrophages of the red pulp.

Lymph Nodes: Both mesenteric and mediastinal lymph nodes show well developed cortical lymphoid tissue and somewhat nodular arrangement but without the formation of actual follicles with secondary centers. There is an abundant amount of hemosiderin in macrophages of the medullary portion of the nodes. This is more marked in the mediastinal nodes which also show prominent mast cells which are not seen in the mesenteric node.

Femoral Bone Marrow: The bone marrow is slightly hypoplastic, hemopoietic cells occupying approximately 75 per cent of the available marrow space in the epiphysis but only about 60 per cent in the metaphysis and the shaft, the remaining space being occupied by fat cells. Erythropoiesis is approximately equal with granulocytopoiesis in extent, and megakaryocytes are quite numerous.

Testes: In the majority of tubules only the basement membrane is well preserved and the lumina are filled with eosinophilic material of stringy or fibrillary appearance through which are scattered round or oval nuclei of somewhat vesicular appearance often with condensation of chromatin in the form of a central bar. Presumably these nuclei represent desquamated Sertoli cells. A few of the tubules contain a normal layer of spermatogonia and spermatocytes but no spermatids or spermatozoa. The interstitial cells appear normal in number and appearance.

Liver: Shows fine fat droplet and occasional confluent fatty infiltration of liver cells scattered irregularly throughout the parenchyma. There is no fatty infiltration of the heart muscle.

Gut and Kidney: Section of gut and kidney show no lesions.

(b) Pathology No. 58864 — LOBUND No. 53 H 7-2A #618
Germ-free - 600 r

Spleen: The Malpighian follicles are quite inconspicuous. The cells forming the remnants of the follicles have partly pyknotic and partly vesicular nuclei, but the absence of phagocytosis suggests that some of these changes may be agonal or postmortem. The outer zone of larger lymphocytes or reticulum cells is completely absent. The red pulp shows numerous macrophages laden with hematogenous pigment and absence of definite myelopoiesis. There are, however, several nests of round cells some of which are dark staining nuclei somewhat resembling normoblasts which may conceivably represent an attempt at erythropoiesis.

Lymph Nodes: The cortical lymphoid tissue of the mesenteric nodes is flattened to a narrow band and the medullary cords are collapsed. The dilated peripheral and medullary sinuses are filled partly with red cells and partly with macrophages containing both erythrocytes and hemosiderin. There is no evidence of active lymphoiesis. The femoral bone marrow is almost entirely fatty. The interstices between the fat cells contain a fair number of macrophages laden with hematogenous pigment as well as a small number of round cells with a moderately dense nucleus and an ample amount of cytoplasm. These cells have not been identified. They might conceivably represent erythroblasts but no definite evidence of erythropoiesis is present. Megakaryocytes, myelocytes, and normoblasts are absent.

Testes: Many tubules contain spermatocytes, spermatids and spermatozoa, but spermatogonia are almost entirely lacking and there is only a very rare mitosis in the layer of spermatocytes. Many tubules show more advanced changes with almost complete loss of spermatogenic elements and only remnants of degenerated cells in an eosinophilic matrix.

Liver: There is fatty infiltration of virtually all liver cells except for a narrow zone around the portal triads and some centrolobular areas with marked atrophy of liver cells and prominent Kupffer cells. The fatty infiltration is primarily small droplet in character but occasionally confluent with sudanophilic material throughout the cytoplasm. There is fatty infiltration of heart muscle cells by small fat droplets.

Pancreas: The pancreas shows considerable postmortem changes but no evidence of intravital lesions. The same applies to the stomach.

Kidney: The kidney shows no lesions.

(c) Pathology No. 58866 — LOBUND No. 53 H 7-2A #633, Germ-free - 600 r

Testes, Heart, Liver, and Spleen: Answer approximately the same description as No. 58863, except that the spleen shows only very little erythropoiesis.

Bone Marrow: The bone marrow shows normal cellularity and numerous megakaryocytes with granulocytopoiesis equal to or exceeding erthropoiesis. The activity of the bone marrow is estimated to be within normal limits, 80% or more of the marrow space being occupied by hemopoietic tissue.

(d) NIH Pathology No. 58867 — LOBUND No. 53 H 7-2A #634, Germ-free - 600 r

Liver, Heart, Bone Marrow, Testes: Are similar to No. 58864. The fatty infiltration of the heart is quite comparable but possibly fatty infiltration of the liver is less in the present rat than in 58864. Of the bone marrow only part of the epiphysis and metaphysis are sectioned and these show purely fatty marrow. There is some interstitial hemorrhage in the testes.

Lymph Nodes: One subcutaneous and one cervical lymph node show similar atrophy of lymphoid tissue and red cell filled sinuses as No. 58864.

Lungs: Show no lesions.

(e) NIH Pathology No. 58865 — LOBUND No. 53 H 7-2B #819
Conventional - 600 r

Spleen: The appearance is quite similar to that of number 58864. Here again the nests of round or oval cells with dark staining nuclei cannot be identified with certainty but a number of them are plasma cells.

Lymph Nodes: None submitted.

Femoral Bone Marrow: The bone marrow is almost entirely fatty. It does not contain an appreciable amount of hemosiderin and macrophages as did No. 58864, and there are virtually no cells in the interstices except in one area suggestive of a possible attempt at myelopoiesis.

Testes: The testes is similar to No. 58864, except that there are fewer tubules with advanced disintegration of spermatogenic cells and there are a few more mitosis in spermatocytes.

Liver: The liver shows a similar fatty infiltration as 58864, except that in the centrolobular areas there is early necrosis rather than simple disappearance of liver cells as evidenced by the intense eosinophilic staining and the pyknotic nuclei.

Heart: The fatty infiltration of the heart muscle is much less prominent than in animal No. 58864.

Adrenal: The glomerulosa is very narrow and contains little lipid material.

Lung: There is some apical emphysema and a minute area of probably chronic pneumonic process, perhaps a focus of lipid pneumonia.

Kidney: The kidney shows no lesions.

(f) NIH Pathology No. 58868 — LOBUND No. 53 H 7-2A #817
Germ-free - 800 r

Spleen: The spleen consists entirely of collapsed reticulum and fibrous tissue in which the outline of follicles are barely perceptible. There is a moderate amount of iron positive pigment scattered throughout.

Thymus: The thymus shows incomplete regeneration of the cortex and a medulla severely depleted of lymphocytes.

Bone Marrow: The bone marrow is entirely fatty, both in the epiphysis and the metaphysis and shaft.

Testes: The tubules are lined by a regular layer of spermatocytes, spermatid and spermatozoa that contain no spermatogonia and only occasional mitoses in the spermatocytes.

Lung: There is a moderately extensive hemorrhage into the lung.

Adrenals: The glomerulosa is quite inconspicuous and hardly to be differentiated from the fasciculata.

Liver: The liver shows a marked fatty infiltration described for No. 58867. The atrophy of the central portion of the liver lobule is marked with transformation of the central portion of the trabeculae into homogeneous strongly eosinophilic masses with only occasional outlines of remnants of nuclei.

Heart: The heart muscle shows patchy fatty infiltration similar to No. 58867.

(g) NIH Pathology No. 58869 — LOBUND No. 43 H7-2B #818
Germ-free - 800 r

Thymus: The thymus is similar to No. 58868. There is some hemorrhage in the interstitial tissue of the pancreas.

Lymph Nodes: The mediastinal lymph node shows virtually complete atrophy of all lymphoid elements, hemorrhage into some of the sinuses and periapsular tissue and prominent mast cells.

Adrenals: The adrenal shows a distinct glomerulosa which, however, is considerably narrowed in several places.

Lung: The lung shows no lesions.

Liver: The liver is similar to No. 58868. The centrolobular portions of the trabeculae, though markedly eosinophilic and somewhat shrunken, still show definite although pyknotic nuclei.

Heart: The heart muscle shows only moderate fatty infiltration.

Bone Marrow: The bone marrow consists entirely of fat cells.

Epididymis: Interstitial hemorrhage, spermatozoa and large degenerating cells present.

(h) General Comment

Animals #617 and 633 show evidence of regeneration of spleen, bone marrow, lymph nodes and testes. The rest of the animals (#618, 619, 634, 817, 818) show continued aplasia of the marrow, and no evidence of lymph node or splenic regeneration, whether they were germ-free or not.

The only difference between conventional animals (#819 and 818) and germ-free animals (618, 634, and 817) is the greater fatty infiltration of the heart muscle in the germ-free ones. Compatible with the concept that germ-free animals had developed a severer degree of anemia at the time of death, with conventional animals succumbing

to infection at an earlier date, while the germ-free animals survived somewhat longer until hemorrhage and cessation of red cell production led to a degree of anemia incompatible with life.

There was evidence of either recent or older hemorrhage in every animal in which lymph nodes were available for study.

(Brecher)

FEDERAL SECURITY AGENCY . Public Health Service
NATIONAL INSTITUTES OF HEALTH . Bethesda 14, Md.

July 21, 1952

Dr. H.A. Gordon
Chief Pathologist
University of Notre Dame
Notre Dame, Indiana

Dear Dr. Gordon:

I find that the guesses I gave you on the survival of the recent batch of rats were in error, due to my having overlooked regenerating tubules in #617 and 600-33.

My corrected, though not necessarily correct or final, guesses are as follows:

		Correct survival time:	
#617	at least 7-8 weeks	62 days	
#618	1-2 weeks, probably nearer 2	17 days	
#619	1-2 weeks, probably nearer 1	18 days	LOBUND insert
#600-33	at least 7 weeks	62 days	
#600-34	1-2 weeks, probably nearer 2	16 days	
#817	1-2 weeks, probably nearer 1	10 days	
#818	not yet examined	12 days	

It was a great pleasure having you visit here, and I am looking forward to future meetings here or in Notre Dame.

With kindest regards.

Yours very sincerely,

George Brecher, M.D.

Encl.
GB:lmy

IV. ADDENDA

1. A Study on the Effects of Total Body Radiation on Contaminated, Previously germ-free Rats

A. Streptococcus Group

This group of two animals, reported on at length in our earlier report (pp. 44-49), was found to be contaminated with a slow-growing anaerobic streptococcus which could not be identified with any species described in Bergey's manual. The rats were first generation germ-free and the maximum time contaminated was 16 days. It was decided that exposure of one of them 800 r total body x-irradiation was warranted and that the other should be held as a control animal.

Accordingly, rat #815 was exposed to 760 r (not 740 as reported earlier) of total body x-irradiation. Unfortunately the rat was too large to fit our standard restraint and as a result the irradiation was done with cotton toweling used as a restraint. The rat was sacrificed 34 days after irradiation. Gross autopsy findings were reported earlier but we include the histological findings in this report.

In view of the unusually long survival time of rat #815 it was felt that it would be worthwhile to investigate the effects of total body x-irradiation on any additionally available mono-or dicontaminated, previously germ-free animals.

NIH. #56437 LOBUND #815

Bone Marrow: There is severe hypoplasia of the marrow, but evidence of regeneration is seen in several areas in the form of islands of hemopoiesis, particularly along the endosteum of the shaft of the femur and at the epiphiseal-metaphyseal junction. However, mature granulocytes and megakaryocytes are very rare. Accumulations of small numbers of apparently hemopoietic cells, usually arranged in triangular shape, are present in scattered areas between fat cells which constitute the bulk of the marrow both in the femur and in the flat bones of the skull. Mast cells are fairly numerous and rather conspicuous in the fatty marrow.

Skeletal Muscle: Normal.

Testes: The seminiferous tubules are frequently widely separated by lightly staining, homogeneous material resembling edema fluid, in which an approximately normal number of interstitial cells are present. The tubules are usually lined by a single layer of spermatogonia, but occasionally spermatocytes of the first order are also present, suggesting very early regeneration, even though mitotic figures in the spermatogonia could not be demonstrated. Later stages of spermatogenesis are absent, except for poorly preserved remnants of spermatozoa in a few tubules.

Liver: Fat stains show small droplet fatty infiltration of a moderate number of individual liver cells distributed irregularly throughout the parenchyma.

Spleen: There is excellent regeneration of the Malpighian follicles which are slightly larger than normal. The red pulp is filled with confluent areas of active erythropoiesis and accumulation of stem cells suggesting early

granulocytopoiesis, but no mature granulocytes are seen, and megakaryocytes are extremely rare.

Adrenal: The glomerular zone appears to contain an ample amount of fat as far as this can be judged from the H & E stained section. The general architecture appears normal.

Lung: There is some emphysematous dilatation of several groups of alveoli. Two subpleural alveoli contain islands of metaplastic bone, possibly representing the end-stage of organized pneumonia.

Brain: Thyroid, parathyroid, and hypophysis appear normal.

Stomach, small and large gut: Well preserved mucosa and muscular wall without any demonstrable lesions.

Comment: The large Malpighian follicles indicate good recovery of lymphopoiesis. There is little evidence of granulocytopoiesis in either the marrow or the spleen, but erythropoiesis is quite marked in the spleen, though as yet very slight in the bone marrow. The appearance of the testes is suggestive of very early regeneration, suggesting that about five weeks have elapsed since irradiation. Although we have no experience with the LOBUND strain of rats, and any conclusions reached on one animal of a new strain can be only speculative, the impression is that an animal with such minimal recovery of bone marrow activity at this late stage must have had a long period of virtually complete bone marrow aplasia, and that an animal reared in a normally contaminated environment would probably not have survived. The striking feature of extensive erythropoiesis in the spleen with very slight bone marrow activity is reminiscent of the early stages in the spleen protected mice and represents a baffling problem.

NIH #36436 LOBUND #816

Lung: Extreme dilatation of pulmonary vessels, marked edema filling nearly all of the air spaces.

Brain: Extreme dilatation and congestion of vessels.

Liver: Marked dilatation of central veins which are filled with blood.

Kidney: Marked dilatation and congestion of veins, with one questionable area of hemorrhage, possibly agonal.

Spleen: Moderately active, relatively small Malpighian follicles, probably normal for the age of the animal. Islands of erythropoiesis and rare granulocytes, as well as an occasional megakaryocyte, are seen in the congested red pulp, which contains a considerable amount of hemosiderin pigment.

Small and Large Gut: Mucosa poorly preserved, possible due to circumstances of death.

Comment: All findings are compatible with death from accidental overheating.

FEDERAL SECURITY AGENCY . Public Health Service

NATIONAL INSTITUTES OF HEALTH . Bethesda 14, Md.

April 3, 1952

Professor James A. Reyniers
Lobund Institute
Notre Dame, Indiana

Dear Professor Reyniers:

Thank you for your kind letter of March 11.

I am very happy to know that radiation studies on the germ-free animals are progressing well, and that I will hear further from Dr. Gordon.

I apologize for the delay in forwarding to you the enclosed reports on Lobund rats #815 and 816. I know you are familiar with the vicissitudes of pursuing various projects at the same time, and I hope you will accept my assurance that this delay was not occasioned by any lack of interest in the germfree animals. I still believe that infection is the major cause of death in the acute syndrome, and that the experiments on germ-free animals appear to represent the most tangible hope of settling this question.

Yours sincerely,

George Brecher, M.D.

cc - Dr. Cronkite

GB:lmy

B. Casecolyticus-Like Micrococcus Group.

As a result of routine bacteriological checking, a group of six previously germ-free rats were found to be contaminated with a Micrococcus described as being similar to Micrococcus casecolyticus but not acids-proteolytic in litmus milk. (The germ-free rats are known to have been contaminated for a maximum of 28 days and a minimum of 15 days prior to irradiation, the last germ-free cultures having been taken 9 April 1952 and the first contaminated culture having been taken on 22 April 1952) These rats and six conventional rats were exposed to 800 r total body x-irradiation using our standard procedures, the conventional animals being irradiated two days prior to the exposure of the previously germ-free rats. The results of this experiment are summarized in Table III.

TABLE III

The Effect of 800 r Total Body X-Irradiation on Monocontaminated (Casecolyticus-Like Micrococcus), Previously Germ-Free Rats

Previously Germ-Free			Conventional Controls		
Number	Date Exposed	Survival Days	Number	Date Exposed	Survival Days
847	5/7/52	2	840	5/5/52	5
848	5/7/52	12	841	5/5/52	8
849	5/7/52	14	842	5/5/52	8
850	5/7/52	14	843	5/5/52	10
851	5/7/52	29	844	5/5/52	10
852	5/7/52	< 1*	845	5/5/52	14
Average Survival Days			Average Survival Days		
16 (Excluding #852)			9		
(11.8 Day including #852)					

* Not found dead upon removal from restraint

C. Bacillus Cereus Group

A group of three animals maintained germ-free for 127 days were found to have become accidentally contaminated with B. Cereus. After these animals were contaminated for a maximum of 36 days they were exposed to 800 r total body x-irradiation in order to investigate the effect of B. Cereus on survival time under these circumstances. Our standard procedures were used to irradiate the rats.

A group of three conventional rats were exposed to 800 r two weeks later. The difference in irradiation dates for the two groups was necessary in order to allow the conventional animals to become habituated to the supplemented diet L-128*.

Of the previously germ-free rats, one animal died 9 days following x-irradiation, another was sacrificed while moribund (in order to insure prompt securing of blood for bacteriological culture) on the 9th day, and the third was sacrificed moribund on the tenth post-irradiation day.

The conventional animals irradiated as controls for this group died on the 6th, 7th, and 8th post-irradiation days.

* Maintained on diet L-128 supplemented with 20 gm/Kg "B-Y-21", consisting of dried grain and skim milk fermentation solubles.

Summarised, the survival data are as follows:

Contaminated		Conventional	
Rat No.	Days Survival	Rat No.	Days Survival
809	9 (died)	853	6 (died)
810	9 (sacrificed moribund)	854	8 (died)
811	10 (died)	855	7 (died)
	(Average - 9.3 days)		(Average - 7 days)

In an effort to establish whether or not the previously germ-free, B. Cereus contaminated rats were bacteremic, cultures were taken of hearts from rats #809, 810, and 811 as well as a sample of venous blood from the abdominal vena cava at the level of the renal veins of rat #811. No positive cultures were obtained from the blood aspirated from the animals' hearts; however, Bacillus cereus was isolated from the caval blood sample taken from animal #811. Inasmuch as no cultures were taken from the peritoneal cavity it is impossible to say with certainty that a bacteremia was involved since there is a possibility that the organism may have been picked up from a contaminated peritoneal cavity.

Macroscopically rat #811 showed evidence of an infectious process in the urinary bladder and if this were an ascending infection, the presence of the contaminant in the caval blood might be accounted for if the heart's blood had not been sterile. The other animals of this group showed no unexpected changes at autopsy.

D. Micrococcus Ureae Group

Two rats of our 4th generation germ-free stock, known to have been germ-free for a minimum of seven days, at which time the dam was killed in the catastrophy of November 14, 1951* which more than decimated our germ-free colony. These rats, then, were mother-suckled for the first seven days and then hand-fed through weaning. Cultures taken of the cage and animals on December 17, 1951 proved to be contaminated with Micrococcus Ureae and Micrococcus (species unidentified). It was decided to expose the rats to an inordinately severe dose of total body x-irradiation, and accordingly these and two conventional rats were exposed to 2000 r X-ray on January 31, 1952 using our standard procedures. There was a very slight difference in the survival time of the conventional rats and the previously germ-free rats, that of the conventional rats being 4 days (both dying without interference) and that of the germ-free animals being 5 days (one dying without interference and the other being sacrificed while moribund for bacteriological study). The bacteriologist' report on the results

* Previously reported on December 17, 1952. Rat #816 (see histology reports) was one of the animals destroyed by heat at that time.

of blood culture taken from the moribund rat is submitted herewith:

Rat #2000 - 2? (151 grams) which had received 2000 r X-ray irradiation was sacrificed by exsanguination (heart puncture) on February 5, 1952. One ml heart blood, taken aseptically in a sterile syringe was inoculated into 10 ml fluid thioglycollate medium and incubated at 37° C.

The culture was examined 1 day, 3 days, 7 days, 20 days, and 48 days after inoculation but no evidence of bacterial growth was detected.

2. Exploratory Experiments

A. The Effect of Post-Irradiation Streptomycin Treatment on Germ-Free Rats.

Eight surplus germ-free and six conventional rats were exposed to 700 r total body x-irradiation under the same physical conditions described in the Method and Materials section of this report. Six of the germ-free animals and the conventional animals were irradiated in pairs. One member of each pair received daily by subcutaneous injection 2.5 mg/100 gm body weight of cathode-ray sterilized streptomycin sulfate* while the other member of each irradiation pair similarly received .025 ml/100 gm body weight of non-pyrogenic 0.9% NaCl solution. The streptomycin solutions were prepared fresh daily in a 100 mg/ml concentration. The two remaining germ-free animals were exposed to a placebo irradiation (kept in the restraint in the exposure cage) for an identical amount of time as the irradiated animals and were treated with streptomycin in the same manner as the irradiated rats. The treatments were started 24 hours following the irradiation of the exposed animals. Table IV presents the results of this study from the viewpoint of survival time. The non-irradiated streptomycin treated rats showed no deleterious effects and at the time this report is written are alive. On the other hand, the irradiated streptomycin treated animals did not differ from their saline treated irradiation partners in terms of behavior or appearance. Rats #701 and 702, described in an earlier portion of this report, are included as non-injected, irradiated, germ-free controls; we deem them valid controls inasmuch as they were irradiated on the same day as the animals in the antibiotic study.

TABLE IV

The Effects of Parenterally Administered Streptomycin Sulfate on the Survival Time of Germ-Free Rats Exposed to 700 r Total Body X-Irradiation*

Saline treated + X-Ray		Streptomycin + X-Ray		X-Ray Only		Streptomycin Only	
Rat #	Days Survived	Rat #	Days Survived	Rat #	Days Survived	Rat #	Days Survived
710A	11	710T	10	701	13	1-T	Survived
711A	11	711T	9	702	16	2-T	Survived
712A	10	712T	9				

* Rats with similar numerical designations, e.g. 710A and 710T, were irradiated simultaneously, as were numbers 701 and 702.

* Graciously provided by Dr. L. Michaud of Merck & Company and sterilized through the courtesy of Dr. E.M. Weber of Charles Pfizer & Company, Brooklyn, New York.

At autopsy no outstanding differences were noticeable macroscopically between the saline treated-irradiated and the streptomycin treated-irradiated rats. It was observed, however, that the injected rats, without exception, showed evidence of considerable hemorrhage into the subcutaneous areas at the sites of the injections. Rats #701 and #702 did not suffer the additional insult of the handling, struggling, and attendant trauma associated with the injection procedures and this may well account for their slightly longer survival time.

B. Concerning Latent and Symbiotic Viruses in Germ-Free Animals

We have felt for some time that a level of justifiable certitude has been attained as regards the bacteria-freeness of our animals. Only recently, however, have we begun to give serious attention to the possible existence of non-symptomatic viral forms in these same bacteria-free animals.

This question of the possible presence of inapparent virus, or viruses, in the "germ-free" animal finds a parallel in the problem of the fate of the viral agent in the ordinary latent or subclinical infections so common to the experience of the bacteriologist and the epidemiologist.

The question of a virus cause of cancer also parallels our basic investigation. We refer to the hypothesis that possibly presently unknown viruses, supposedly indigenous or only temporarily resident in the animal hosts, may become cancer-inducing when provoked by the right chemical substance or physical stress. Just as we do not yet know whether our germ-free animals do or do not harbour non-symptomatic viruses, so perhaps is it unknown but what the latent, unprovoked virus may not be the key to the cancer enigma.

The foregoing are exemplicative of problems of real practical significance which are yet strictly corollary to our own rather fundamental interest. Whether pure or applied, however, all of these problems are equally adamant to direct attack. There is, in fact, a suggestion of the prodigal even in the thought of attacking them, and this, being evident to research administrators, probably accounts in some measure for the paucity of available experimental data on such occasions when the subject is turned to. Accordingly, though the importance of the question is recognized, report at this time must be limited to certain incidental findings shaved from other currently more extensive undertakings.

(a) Attempt at X-Ray Activation of Latent Viral Agents in Germ-Free Chicks.

Exp. No. 59L2-1 Attempt at X-ray stimulation of latent Rous sarcoma virus in GF chicks.

Protocol A. (Birds from Exp. No. 47L2-3)

GF Chick #651. Inoc. IM with 1.0 ml of active Rous Filtrate #VI but found to be resistant to it under 73 days of observation.

Conventional Chick #III-3. Developed an atypical tumor to 0.5 ml of Filtrate #VI, but this then showed complete regression prior to X-ray exposure (as noted below).

Conventional Chick #III-6. Inoc. with 1.0 ml of normal chicken muscle hash and maintained in close contact with Rous tumor-bearing chicks for a period of 73 days prior to X-ray exposure (as noted below).

Irradiation. These three chickens were treated with 420 r of X-rays. Observation was made for 31 days after exposure with no external sign of tumor induction during this period. The birds were then sacrificed. Necropsy disclosed no pathology suggestive of neoplasia.

Protocol B. (Birds from Exp. No. 47L2-4)

GF Chick #381. Inoc. with 0.5 ml of active Rous Filtrate #1X, but failed to respond after 57 days, at which time irradiation was made.

GF Chick #384. Not inoculated but housed under GF conditions in close contact with Rous tumor-bearing birds for a period of 57 days, and then irradiated.

Irradiation. These two chicks treated with 300 r of X-rays after becoming contaminated. No external signs of tumor 90 days after irradiation.

Exp. No. 59L2-2 Attempt at X-ray stimulation of latent human infectious hepatitis virus in GF chicks.

Protocol A.

Purpose: To attempt to arouse latent infectious hepatitis virus in GF chicks, previously inoculated intraperitoneally and intravenously, without effect. Chicks of the same clutch and housed in the same GF unit, but inoculated intramuscularly or administered the same virus ultrafiltrate orally, became ill and showed the "usual syndrome" as previously observed with this virus in GF chicks.

Methods: Three chicks were involved.

GF chick #439 (♀) inoculated IP with 0.25 ml of filtrate
GF chick #437 (♂) inoculated IV with 0.25 ml of filtrate
GF chick #438 (♂) inoculated IV with 0.25 ml of filtrate
These were Highview Farm chicks, inoculated at 28 days of age. The three chicks listed were exposed to 330 r of X-rays 55 days after inoculation with the virus.

Results: Two GF chicks inoculated IM and one GF chick receiving an oral administration of this same virus preparation became ill in about 9 or 10 days and all three grew moribund within 13 days after the administrations of the virus. The three GF chicks inoculated IP and IV, listed above, showed no definite signs of illness over a period of 55 days following inoculation with the virus. At this time these three GF chickens were irradiated with 300 r of X-rays. Observation of these chicks was continued for a further 35 days. No untoward signs were detected and necropsy, performed 90 days after the inoculations, disclosed no pathology attributable to virus or X-ray effects.

(b) Attempt at X-ray Activation of Symbiotic Viral Agents in Germ-Free Chicks.

Exp. No. 59L3-1 Attempt at X-ray stimulation of possible symbiotic viral agents in GF chicks.

Protocol A

Purpose: To test the possibility of activation of agents or of production of toxic products in GF chicks by X-ray irradiation, such activation or production to be determined by serum transfer from irradiated GF chick(s) to normal GF chick(s).

Method: GF chick #14 was irradiated with 400 r of X-rays. Twenty-four hours after exposure heart blood was drawn from this chicken. Since this work was done in a GF unit, Serum A was obtained by allowing the RBC to settle out under a force of 1 g. A second portion of this blood, however, was removed from the GF cage and centrifuged on the outside to yield Serum B. Some of Serum A was also removed to the outside.

Inoculations: (1) GF chick #18 was inoculated intravenously with 0.5 ml of Serum A (containing some RBC).
(2) Conventional chick #18B was inoculated intravenously with 0.5 ml of Serum A (containing some RBC)
(3) Conventional chick #18C was inoculated intravenously with 0.5 ml of Serum B (clear).

Results: No signs of illness were detected in any of these four birds during a 29-day period of observation. All of the chicks appeared active and normal throughout the whole of this time.

The results of these few fragmentary experiments of course do not point to any standard application of X-ray irradiation to the activation either of known subclinical viral infection or of presently unknown symbiotic viruses. This is for the future. We feel that the development of a method or methods for the detection of inapparent viruses (latent and/or symbiotic) is of prime importance to our own ends and to the practical purposes cited at the beginning of this section. We expect to do further work in this direction.

C. Post-Mortem Bacterial Invasion into the Peritoneal Cavity of the Conventional Albino Rat.

The question as to the reliability of taking bacteriologic cultures from the tissues or parenteral cavities of animals which have been dead for varying lengths of time is an important one since it is often desireable to establish whether death was caused by, or at least coincident with bacteremia. Obviously, the tissues and parenteral cavities of a dead animal can be expected to show eventual contamination via bacterial invasion from the intestinal tract etc. In a preliminary experiment, data are presented to indicate an approximation of time at which post-mortem bacterial invasion takes place into the peritoneal cavity of the conventional albino rat.

Fifty-two adult white rats in apparent good health were sacrificed by an overdose of ether. The time of death was arbitrarily set at the cessation of breathing. The animals were kept at room temperature for various lengths of time following sacrifice.

The culture technique is briefly summarized:

1. Immerse total body in 0.1% potassium mercuric iodide to wet down surface.
2. Incise and fold back abdominal skin layer with electric cautery.
3. Paint exposed abdominal muscle layer with tincture of iodine.
4. Incise muscle layer and peritoneal lining with electric cautery.
5. Sample peritoneal cavity with sterile cotton applicator swab.
6. Culture swab in fluid thioglycollate medium at 37°C and observe for growth.

Results

The case distribution of positive and negative peritoneal cultures is shown for varying time intervals after death. (see graph).

With rare exception, animals dead less than ten hours showed negative peritoneal cultures. At the later time intervals, the positive culture cases increased but even at the 18.75 hour mark, a negative culture was still encountered.

The contamination was generally made up of a mixed bacterial population involving Gram positive to Gram variable sporulating rods, Gram negative rods and Gram positive cocci.

While at this writing, we have not established the time at which 100% contamination can be expected, a 50% contamination expectancy probably lies in the 10-15 hour range.

Assuming the bacteria eventually found in the peritoneal cavity to be the result of invasion from the intestinal lumen, the intestinal wall of the normal conventional rat apparently can contain its microflora for an appreciable time after death.

Blood cultures were also checked in eight of the rats reported above, in those cases where heart blood could still be obtained aseptically by

needle and syringe. Although most heart blood samples were obtained in rats cultured at the earlier post-mortem times, it was possible to obtain heart blood from a rat dead for 15-1/2 hours. All heart blood cultures were sterile. It was later established that all blood cultures came from rats which had also shown negative peritoneal cavity cultures.

Supplement

Several conventional laboratory rats which had died spontaneously after X-ray irradiation were examined bacteriologically by culturing the heart blood and peritoneal cavity.

Case I: Rat 53H8-2B #601

Status: Conventional laboratory rat
X-ray: 9/30/52 (600 r)
Died: 10/18/52 (18 days post X-ray) spontaneous death
Cultures: taken 1 hour after death
Peritoneal cavity: sterile
Heart blood: Gram-negative rods

Case II: Rat 53H10-1-2B #703

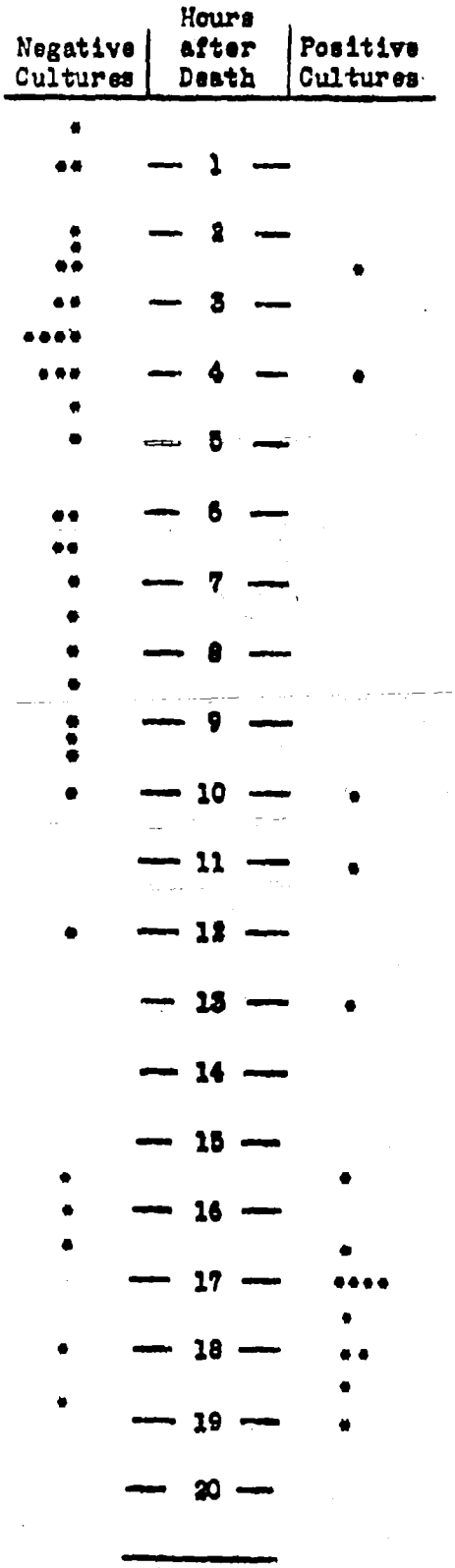
Status: Conventional laboratory rat
X-ray: 11/6/52 (700 r)
Died: 11/13/52 (7 days post X-ray) spontaneous death
Cultures: taken 4 hours after death
Peritoneal cavity: sterile
Heart blood: Gram-negative rods

Case III: Rat 500E (Hypervitaminosis E)

Status: Conventional laboratory rat fed high vitamin E diet
X-ray: 9/28/52 (800 r)
Died: 10/4/52 (6 days post X-ray) spontaneous death
Cultures: taken 15 minutes after death
Peritoneal cavity: Gram-negative rods
Heart blood: not run

In the three cases sited for X-rayed conventional laboratory rats, Cases I and II showed sterile peritoneal cultures taken 1 and 4 hours after death respectively, whereas Gram-negative rods were recovered in both cases from heart blood. In Case III, the peritoneal culture taken 15 minutes after death resulted in recovery of Gram-negative rods. Heart blood was not taken on this latter animal but it is probable that with a positive peritoneal culture, the blood culture would also have been positive in this particular case.

Distribution of Positive and Negative Peritoneal Cultures Taken at Various Time Intervals After Death of Normal Rats.



Note: Each dot represents one animal examined at the indicated time period following death.

Figure 8

3. Meeting of the LOSUND-Atomic Energy Commission Advisory Board, December 17, 1951, 9:00 A.M.

This meeting was attended by Dr. LeRoy, Commander Cronkite, Dr. Miller, Dr. Tuttle, Professor Reyniers and Dr. Gordon.

1. Reyniers and Gordon presented the results of the rat irradiation experiments to the group. In essence, there was a general agreement in respect to the interpretation of the findings (see attached report).

2. The group accepted Dr. LeRoy's plan for the next radiation experiment. This was laid out to establish definitely the lethal dose and survival time in the germ-free rat. Details are as follows:

IRRADIATION	GERM FREE RAT	CONTROL
500 r	8	8
600 r	8	8
700 r	8	8
800 r	8	8
900 r	8	8
1000 r	8	8
TOTAL	48	48

In order to obtain significant results, it was emphasized by the advisors (especially by Dr. LeRoy and Dr. Tuttle) that the experiment should not be drawn out over a longer period of time: comparability within each group and between the two experimental groups (germ-free and control) suffers considerably under such condition. It was also made clear during the discussion that our results are urgently needed by the A.E.C. to plan adequately future tests.

The group accepted the following plan: 3 irradiation days per week with 2 germ-free and 2 control rats per day, the total of 12 animals per week. According to this, the experiment would be completed within 8 weeks.

Other details: The rats should weigh 200 - 250 grams and should be 10 to 14 weeks old. Constancy in bodyweight, however, is more important than constancy in age. As it has been shown recently (greenhouse on 5000 mice) that sexes do not respond differently to radiation, no restrictions were made along these lines.

All animals should be kept on the same diet. The controls should be put on the sterilized ration as soon as possible.

All animals should be observed either until they become moribund or until death occurs. Recovered survivors should be sacrificed not sooner than 60 days after radiation. Autopsy results, organ weights and terminal hematological data should be recorded when possible. Hematology during the course of the experiment will be omitted.

Bacteriology laboratory should perform only routine tests for germ-freeness.

3. Another decision reached by the group was to irradiate any notobiotic rats (intentionally or accidentally mono- or poly- contaminated) as they approach the mentioned weight and age. The radiation dosage should be uniform 800 r. When moribund, attempts should be made to obtain heart blood cultures in thioglycollate. All other details of this experiment should be the same as described under #2.

4. Scruggs presented the findings of the preliminary chicken irradiation experiment. There were no special comments made.

ADDENDA

A. After the closing of the formal meeting, Dr. Cronkite and Dr. Tuttle suggested to fortify the LOBUND normal control LD50 data by the irradiation of additional 30 animals at 600 and 700 r (15 animals in each group). Another suggestion made at this time was to expose one germ-free rat to 2000 r dosage and "see what will happen". According to them, a survival longer than 6 to 8 days in this case would be a real "jackpot".

B. When possible spot-check platelet counts should be made in whole blood diluted with 1% ammonium oxalate.

C. Dr. LeRoy promised to send to LOBUND the blueprints of an efficient rat restraining device as well as handy blood micropipettes.

The meeting closed around 6:00 P.M.

V. GENERAL SUMMARY

1. Various effects of X-irradiation have been studied in rats and chickens maintained germ-free and in conditions of controlled contamination.

2. Clinical observations. In the range of 400 r or less there was no difference between germ-free and conventional control rats.

In animals exposed to more than 400 r:

A. Terminal diarrhoea was observed only in 1 out of 6 germ-free rats, while all conventionals were severely affected by it.

B. In germ-free rats the agonal period never lasted longer than 5 hours; the conventional controls lay in a profound shock-like state for as long as 24 hours.

C. Among the germ-free rats the chances of recovery after irradiation seemed to be the same as for the conventional animals. An exception was a narrow zone around 600 r where a differential effect in favor of the germ-free seemed to be present.

D. In the range between 600 r and 1000 r the state of germ-freeness prolonged the life expectancy of the experimental animals. Under these circumstances the survival time of the germ-free was in average the double of the conventional value. However, as mentioned, the end result was invariably the loss of all animals in this range.

E. In respect of bodyweight changes which incur after irradiation certain differences seemed to be present in the medium and high radiation dose groups. Germ-free rats exposed to 700 r maintained their original bodyweight for as long as 8 days after the irradiation and begun their terminal downward slope only after this period; conventional rats seemed to decline in weight quite soon after the X-ray exposure. At the 1000 r level the onset and the rate of bodyweight loss was almost identical between germ-free and conventionals. However, both at 700 r and 1000 r the endpoint of the relative weight loss appeared to be the same in the two examined animal categories.

3. Because of the lack of new hematological evidence, only previous findings are reported.

Hematologically there was no difference in RBC between the germ-free and control 300 r rats. In both 300 r groups RBC was qualitatively the same: however, the critically low values (around 1 million) were reached in about 10 days by the controls and in 22 days by the germ-free.

In both control groups (300 and 800 r) all white elements of the blood sharply fall to a minimum within 3 days; after the 7th day there was either a gradual recovery (300 r animals) or practically no restitution (800 r animals). In the germ-free this pattern was different in some detail: while the absolute lymphocyte count dropped essentially in the same manner as the total white and/or the lymphocytes of the controls, the granulocytes showed a very marked peak of short duration within the first few days after the irradiation. This peak was most clearly indicated in case of the mature granulocytes, but it was also discernible among the immature forms. In the

case of recovery (300 r) the onset of the restitution of the white elements was also around the 7th day.

4. In respect of gross pathology the overall picture seen in both groups was the same in kind, differing in degree, in that the conditions seen in the conventionals were considerably intensified in the germ-free animals.

5. In respect of histology the significant findings were:

   A. Hemorrhages in germ-free animals (i.e. in absence of infection).
   B. Severer degree of degenerative processes (anemia, fatty degeneration of heart and liver) in germ-free than in conventionals.

6. The presence of a single or double contaminants did not affect uniformly the previously germ-free animal; a relationship seemed to exist between the kind of the contaminant and the life expectancy of the experimental animal. The range of the survival time of these animals was generally between what can be described as the "germ-free and conventional endpoints".

7. After a 2000 r exposure there was practically no difference in survival time between a [illegible] of di-contaminated, previously germ-free rats and their conventional controls.

8. In an exploratory experiment an unsuccessful attempt was made to study the effect of post-irradiation streptomycin-treatment on germ-free rats.

9. A few preliminary experiments were run in normal and irradiated germ-free & conventional chickens to study the possible presence of latent and symbiotic viruses in germ-free animals.

10. In an exploratory experiment data are presented to indicate an approximation of time at which post-mortem bacterial invasion takes place into the peritoneal cavity of the conventional rat.

TABLE V

General Table of Animal Material

No.	LOBUND Code	Sex	Category	Irr. Dose	N.I.H. Code	Remarks
1	53LOB2-3 #814	♂	Germ-free	800	55790	Germ-Free I
2	" 313	♀	"	300	55789	
3	" 807	♀	Conventional	800	55791	
4	" 808	♂	"	800	-	
5	" 809	♀	"	800	-	
6	" 810	♂	"	800	-	
7	" 811	♀	"	800	-	
8	" 812	♂	"	800	55792	
9	" 301	♀	"	300	-	
10	" 303	♂	"	300	-	
11	" 304	♀	"	300	-	
12	" 305	♀	"	300	-	
13	" 306	♀	"	300	-	
14	" 307	♂	"	300	-	
15	53H-7 #1033	♂	Germ-free	1000	-	Germ-Free II
16	" 1034	♂	"	1000	-	
17	" 817	♂	"	800	58868	
18	" 818	♂	"	800	58869	
19	" 833	♂	"	800	-	
20	" 834	♂	"	800	-	
21	" 835	♂	"	800	-	
22	" 836	♂	"	800	-	
23	" 617	♂	"	600	58863	
24	" 618	♂	"	600	58864	
25	" 633	♂	"	600	58866	
26	" 634	♂	"	600	58867	
27	" 333	♂	"	600	-	
28	" 334	♂	"	600	-	
29	" 1035	♂	Conventional	1000	-	
30	" 1036	♂	"	1000	-	
31	" 819	♂	"	800	-	
32	" 820	♂	"	800	-	
33	" 837	♂	"	800	-	
34	" 838	♂	"	800	-	
35	" 839	♂	"	800	-	
36	" 840	♂	"	800	-	
37	" 619	♂	"	600	58865	
38	" 635	♂	"	600	-	
39	" 636	♂	"	600	-	
40	" 335	♂	"	300	-	
41	" 336	♂	"	300	-	

TABLE V (CONT'D)

No.	LOBUND Code	Sex	Category	Irr. Dose	N.I.H. Code	Remarks
42	53H-10-2 #1001	♂	Germ-free	1000	-	Germ-Free III
43	" 1002	♂	"	1000	-	
44	" 701	♂	"	700	-	
45	" 702	♂	"	700	-	
46	" 501	♂	"	500	-	
47	" 502	♂	"	500	-	
48	" 1003	♂	Conventional	1000	-	
49	" 1004	♂	"	1000	-	
50	" 703	♂	"	700	-	
51	" 704	♂	"	700	-	
52	" 503	♂	"	500	-	
53	" 504	♂	"	500	-	
54	53LOB2-3 #815	♂	Contaminated	800	56437	Streptococcus
55	" 816	♂	"	800	56436	Group
56	53H8 #847	♂	Contaminated	800	-	Cancolyticus -
57	" 848	♂	"	800	-	like group
58	" 849	♂	"	800	-	
59	" 850	♂	"	800	-	
60	" 851	♂	"	800	-	
61	" 852	♂	"	800	-	
62	" 810	♂	Conventional	800	-	
63	" 811	♂	"	800	-	
64	" 812	♂	"	800	-	
65	" 813	♂	"	800	-	
66	" 814	♂	"	800	-	
67	" 845	♂	"	800	-	
68	53H9 #809	♀	Contaminated	800	-	B.cereus group
69	" 810	♂	"	800	-	
70	" 811	♂	"	800	-	
71	" 853		Conventional	800	-	
72	" 854		"	800	-	
73	" 855		"	800	-	
74	53H5-2 #2001	♂	Contaminated	2000	-	Micrococcus ureae
75	" 2002	♀	"	2000	-	group
76	" 2003	♀	Conventional	2000	-	
77	" 2004	♀	"	2000	-	

TABLE V (CONT'D)

No.	LOBUND Code	Sex	Category	Irr. Dose	N.I.H. Code	Remarks
78	53H-10-2 #710A	♂	Germ-free saline control	700	-	Streptomycin experiment
79	" 711A	♂	"	700	-	
80	" 712A	♂	"	700	-	
81	" 710T	♂	Germ-free + streptomycin	700	-	
82	" 711T	♂	"	700	-	
83	" 712T	♂	"	700	-	
84	" 1T	♂	"	-	-	
85	" 2T	♂	"	-	-	
86	" 1A	♂	Conventional saline control	700	-	
87	" 2A	♂	"	700	-	
88	" 3A	♂	"	700	-	
89	" 1B	♂	Conventional + streptomycin	700	-	
90	" 2B	♂	"	700	-	
91	" 3B	♂	"	700	-	
92	59L2-1 #351	-	Germ-free	300	-	Latent and symbiotic virus experiment
93	" #384	-	"	300	-	
94	" 651	-	"	430	-	
95	" III-3	-	Conventional	430	-	
96	" III-6	-	"	430	-	
97	59L2-2 #437	-	Germ-free	300	-	
98	" 438	-	"	300	-	
99	" 439	-	"	300	-	
100	59L3-1 #14	-	Germ-free	400	-	
101	" 18	-	"	-	-	
102	" 18B	-	Conventional	-	-	
103	" 18C	-	"	-	-	
104 155	non-irradiated conventional rats					Post mortem bacterial invasion

Summary:

Animals	Germ-Free	Contaminated	Conventional	Total
Rats	30	19	94	143
Chickens	8	-	4	12
Total	38	19	98	155